Crosstalk between the androgen and estrogen receptors in breast cancer

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DECLARATION

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the owner of the copyright thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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SUMMARY

Hormone replacement therapy (HRT) is used by post-menopausal women to alleviate symptoms associated with decreased endogenous estrogen levels, such as hot flashes and vaginal dryness. Although HRT is considered an effective treatment method, results from clinical trials such as those conducted by the Women's Health Initiative (WHI) and Million Women Study (MWS) revealed that HRT usage is associated with increased risk of invasive breast cancer. The mechanism whereby the hormones used in HRT contribute to breast cancer risk is not fully understood. These hormones elicit their biological effects by binding to intracellular steroid receptors such as the estrogen (ER) and progesterone receptor (PR). For many years it was thought that the ER is the main steroid receptor implicated in breast cancer biology, however, data in the literature suggests that crosstalk between steroid receptors play an important role in the development and progression of this disease. Recent evidence suggests that the androgen receptor (AR) can function as a tumour suppressor and thus has the potential of being a prognostic marker and therapeutic target in breast cancer. Given that the AR has been shown to inhibit the transactivation function of ERα, this raised the question of whether the AR would also inhibit the transactivation function of the ERβ subtype. Moreover, considering that ERs have both transactivation and transrepression functions, studies investigating the influence of the AR on the transrepression function of both ERα and ERβ are lacking. This study therefore investigated whether the AR, in the absence or presence of known AR agonists (the natural AR ligand 5α-dihydrotestosterone (DHT)), the androgenic progestins, medroxyprogesterone acetate (MPA) and norethisterone-acetate (NET-A)), could modulate the transcriptional activity of the ERβ. The ER- and AR-negative MDA-MB-231 breast cancer cell line, transiently transfected with expression vectors for either ERα or ERβ and the appropriate promoter-reporter construct, was used as model system. The results showed that the unliganded or liganded AR has no effect on the transactivation function of ERβ on a synthetic estrogen response element (ERE)-containing promoter but downregulates the ERβ-driven mRNA expression of the endogenous PR gene. This study also shows estradiol (E_2)-induced transactivation on a synthetic ARE- and an endogenous androgen response element (ARE)-containing promoter via ERα. For transactivation via ERβ, similar results were only seen on the endogenous ARE-containing promoter. Moreover, we show for the first time that both the unliganded- and ligand-bound AR inhibits the transrepression function of ERα, while interestingly only the ligand-bound AR was able to inhibit the transrepression function of ERβ. No E_2-induced cell proliferation was observed in the MDA-MB-231 cell line overexpressing either ERα or ERβ under the experimental conditions used. In conclusion, the results of this study are in agreement with previous evidence suggesting that the AR inhibits the activity of ERα. The precise physiological implications of these results remain to be determined. Finally, although the results from this study are preliminary and have certain limitations, it nonetheless highlights the fact that the role of the AR in breast cancer is a complex one. Thus, our findings may aid our understanding of crosstalk between the ER and AR signalling pathways, and how it contributes to the growth and survival of breast cancer cells.

OPSOMMING

Hormoon vervangingsterapie (HVT) word deur na-menopousale vrouens gebruik om simptome geassosieerd met verlaagde endogeen estrogeenvlakke, soos warmgloede en vaginale droogheid, te verlig. Alhoewel HVT beskou word as ‘n effektiewe behandelingsmetode, toon resultate van kliniese toetse soos byvoorbeel die Vroulike Gesondheids Inisiatief (VGI) en Miljoen Vroue Studie (MVS) dat die gebruik van HVT ‘n toename in die risiko van indrigende borskanker veroorsaak. Die meganisme waardeur hierdie hormone, wat gebruik word in HVT, bydra tot die risiko van borskanker word nog nie ten volle verstaan nie. Hierdie hormone voer hul biologiese effekte uit deur te bind aan intrasellulêre steroïd reseptore soos die estrogeen (ER) en progesteroon (PR) reseptore. Vir baie jare was dit geglo dat die ER die hoof steroïd reseptor betrokke is in borskanker, maar data in die literatuur stel egter voor dat ‘n wisselwerking tussen steroïd reseptore ‘n belangrike rol in die ontwikkeling en bevordering van hierdie siekte speel. Onlangse bewyse dui daarop dat die androgeen reseptor (AR) as ‘n onderdrukker van gewasse kan funksioneer, en dus die potensiaal het om as ‘n prognostiese merker en terapeutiese teiken in borskanker te dien. Die feit dat die AR al getoon het om die transaktiverings funksie van die ERα te onderdruk, het gelei tot die opwekking van die vraag of die AR ook die transaktiverings funksie van die ERβ subtipe kan onderdruk. Verder, siende dat die ER’s beide transaktiverings en transonderdrukkings funksies het, is dit belangrik om in ag te neem dat daar ‘n te kort is aan navorsing wat die invloed van die AR op die transonderdrukkings funksie van beide ERα en ERβ ondersoek. Die huidige studie het dus ‘n ondersoek ingestel om te bepaal of die AR, in die afwesigheid of teenwoordigheid van bekende AR agoniste (die natuurlike AR ligand 5α-dihidrotestosteroon (DHT), die androgeniese progestiene medroksieprogesteroon asetaat (MPA) en noretisteroon asetaat (NET-A)), die transkripsionele aktiwiteit van die ERβ kan verander. Die ER- en AR-negatiewe MDA-MB-231 borskankersellyn, tydelik getransfekteer met die uitdrukkingsvektore vir óf die ERα óf die ERβ en die toepaslike promotor-rapporteerder konstruk, was gebruik as die model sisteem. Die resultate het getoon dat die AR geen effek op die transaktiverings funksie van die ERβ op ‘n sintetiese estrogeen respons element (ERE)-bevattende promotor het nie, maar dat dit wel die ERβ-gedrewe mRNS uitdrukking van die endogene PR geen onderdruk. Estradiool (E_2)-geïnduseerde transaktivering op ‘n sintetiese androgeen response element (ARE) en ‘n endogene ARE-bevattende promotor deur ERα word ook in hierdie studie aangedui. Soortgelyke resultate is ook waargeneem vir die transaktivering deur ERβ op ‘n endogene ARE-bevattende promotor. Verder, wys ons vir die eerste keer dat beide die ligandlose en ligand-gebonde AR die transonderdrukkings funksie van ERα inhibeer, terwyl interessant genoeg slegs die ligand-gebonde AR die vermoë het om die transonderdrukkings funksie van ERβ te inhibeer. Onder hierdie gebruikte eksperimentele kondisies, was geen E_2-geïnduseerde selproliferasie in die MDA-MB-231 sellyn, wat óf ERα óf ERβ ooruitdruk, waargeneem nie. In samevatting, die resultate van hierdie studie stem ooreen met vorige bewyse wat voorstel dat die AR die aktiwiteit van ERα onderdruk. Die presiese fisiologiese implikasies van hierdie resultate moet nog bepaal word. Ten slotte, alhoewel die resultate van hierdie studie slegs voorlopig is en

sekere beperkinge toon, beklemtoon dit nietemin die feit dat die rol van die AR in borskanker kompleks is. Dus, ons bevindings mag bydra tot die huigelike begrip van die wisselwerking wat tussen die ER en die AR seintransduksiepaaie voorkom, en mag ook help om te verstaan hoe dit moontlik kan bydra tot die groei en oorlewing van borskankerselle.

I would like to dedicate this thesis to my dad, **Thamsanqa Joseph Ndlovu.**

May your soul rest in peace.

Philippians 4:8-9

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To the **LORD, GOD ALMIGHTY**, thank you for keeping me until this day. You are my pillar of strength.

LIST OF ABBREVIATIONS

17β-HSD	17β-hydroxysteroid dehydrogenase
AF-1	activation function-1
AF-2	activation function-2
AI	aromatase inhibitor
ANOVA	analysis of variance
AP-1	Activator Protein-1
AR	androgen receptor
ARA	androgen receptor associated
ARE(s)	androgen response element(s)
ATCC	American Type Culture Collection
AVG	average
bp	base-pair
CDK	cyclin dependent kinases
cDNA	complementary deoxyribonucleic acid
C/EBP	CCAAT enhancer binding protein
CHD	coronary heart disease
ChIP	chromatin immunoprecipitation
Co-IP	co-immunoprecipitation
CP	crossing point
CT	cycle number
C-terminal	carboxy-(COOH-) terminal
CTSD	cathepsin D

CYP	cytochrome P450
DBD	DNA-binding domain
DEPC	diethylpyrocarbonate
DHT	dihydrotestosterone
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
E	exponential amplication value
E_1	estrone
E_2	17β-estradiol
E_3	estriol
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetra-acetic acid
ER	estrogen receptor
ERE	estrogen response element
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
EtOH	ethanol
FCS	fetal calf serum
FRET	fluorescence resonance energy transfer
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GF	growth factor
GnRH	gonadotropin-releasing hormone
GPCRs	G-protein-coupled receptors
GR	glucocorticoid receptor

HCL	hydrogen chloride
HER-2	human epidermal growth factor receptor-2
HRP	horseradish peroxide
HRT	hormone replacement therapy
Hsp(s)	heat-shock protein(s)
ICI 182,780	fulvestrant or faslodex
IκB	inhibitor of kappa B
IKK	IκB kinase
IL-6	interleukin-6
KLK	kallikrein
LB	Luria-Bertani
LBD	ligand binding domain
MAPK	mitogen-activated protein kinase
MIB	mibelorone
MNAR	modulator of non-genomic activity of estrogen receptor
MPA	medroxyprogesterone acetate
MR	mineralocorticoid receptor
mRNA	messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide
MWS	Million Women Study
NET-A	norethisterone / norethindrone acetate
NFκB	nuclear factor kappa-B
NKX3	prostate homeobox
NO	nitric oxide
NRs	nuclear receptors

OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PMA	phorbol-myristate acetate
PR	progesterone receptor
PR-A	progesterone receptor A
PR-B	progesterone receptor B
PRE	progesterone response element
PSA	prostate specific antigen
qPCR	quantitative polymerase chain reaction
RANTES	Regulated on Activation, Normal T-cell Expressed, and Secreted
re-ChIP	chromatin re-immunoprecipitation
RLU	relative light unit
mRNA	messenger ribonucleic acid
RT-PCR	real time-polymerase chain reaction
SARM	selective androgen receptor modulator
SD	standard deviation
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SERD	selective estrogen receptor downregulator
SERM	selective estrogen receptor modulator
SHBG	sex hormone binding globulin
SM	second messenger
Sp1	specificity protein 1
SRC-1	steroid receptor co-activator 1

SRE	steroid response element
SRs	steroid receptors
R	relative expression
TBS	tris buffered saline
TBST	tris buffered saline tween
TFs	transcription factors
TGF-α	transforming growth factor alpha
T_m	melting temperature
TNBC	triple negative breast cancer
TNF-α	tumour necrosis factor alpha
VEGF	vascular endothelial growth factor
WHI	Women's Health Initiative
WHO	World Health Organization

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Chapter 1
Literature Review

1.1. Introduction

Breast cancer accounts for about one fourth of all female cancers, and is the second leading cause of cancer-related death worldwide in women (Sommer and Fuqua 2001, Wang *et al.* 2006, Yu *et al.* 2011, Seok-Jun *et al.* 2012, Chottanapund *et al.* 2013). Several factors have been identified, which increases the risk of developing breast cancer, and include genetics, epigenetics, lifestyle and endocrine therapy such as hormone replacement therapy (HRT) (Bergkvist *et al.* 1989, Black 1994, Colditz *et al.* 1995, McPherson *et al.* 2000, Rossouw *et al.* 2002, Beral 2003). HRT is used by post-menopausal women to alleviate the symptoms associated with menopause, due to decreased estrogen levels. The symptoms of menopause include hot flashes, vaginal dryness, dysfunctional uterine bleeding, urogenital atrophy and psychological symptoms such as irritability, depression and insomnia (McKinlay *et al.* 1992, Ravn *et al.* 1994, Farquhar *et al.* 2009). HRT is given as estrogen alone to women who have undergone a hysterectomy, while women with an intact uterus are administered a combination of estrogen and a progestin, such as medroxyprogesterone acetate (MPA) or norethisterone acetate (NET-A) (Pike *et al.* 1997, Marsden 2003, Verkooijena *et al.* 2009, Mia *et al.* 2013). Progestins are added to reduce the risk of estrogen-induced endometrial hyperplasia (Gelfand and Ferenczy 1989, Pinto *et al.* 2003, Furness *et al.* 2009). Although HRT is considered an effective treatment for relieving menopausal symptoms, results from the Women's Health Initiative (WHI) trial and the 'Million Women Study' (MWS) raised much alarm by showing that the use of HRT (estrogen alone and estrogen plus progestin) leads to increased risk of breast cancer in women (Rossouw *et al.* 2002, Beral 2003). Interestingly, recent studies have shown that there has been a decrease in breast cancer incidence, which has been suggested to be linked to a decline in HRT usage (Katalinic and Rawal 2008, Silvermana *et al.* 2011, Antoine *et al.* 2014). It is thought that the primary cause of the reduction in the use of HRT over the last few years is due to the results of the WHI study (Chlebowski *et al.* 2003, Chlebowski *et al.* 2010, Holm *et al.* 2014). Understanding the mechanisms whereby hormones used in HRT increase

women's risk of developing breast cancer is essential for finding improved therapies for the treatment of breast cancer. It is known that the hormones used in HRT elicit their biological effects primarily via binding to the estrogen receptor (ER) or the progesterone receptor (PR), which are both members of the steroid receptor family (Osborne *et al.* 2001, Zilli *et al.* 2009, Kittler *et al.* 2013, Knutson and Lange 2014). The ER exists as two subtypes namely ERα and ERβ (Brandenberger *et al.* 1997, Enmark *et al.* 1997, Nilsson *et al.* 2001), while the PR is expressed as two isoforms called PR-A and PR-B, respectively (Kastner *et al.* 1990).

Estrogen acting via ERα is considered to be the major etiological factor in breast cancer and stimulates breast cancer cell proliferation in ER-positive breast cancer cells (Jordan and Ford 2011, Wu *et al.* 2012), while ERβ inhibits ERα-mediated cell proliferation (Gougelet *et al.* 2005, Paris *et al.* 2012, Saxena *et al.* 2013). Studies have reported that the PR is also involved in breast cancer cell proliferation (Kalkhoven *et al.* 1994, Saitoh *et al.* 2005, Chen *et al.* 2012), with differential contributions of the PR isoforms being reported (Graham *et al.* 1996, Hopp *et al.* 2004, Stendahl *et al.* 2006, Giulianelli *et al.* 2013). For example, PR-B has been shown to have proliferative effects on breast cancer cell proliferation in the presence of its natural ligand progesterone (Conneely *et al.* 2003, Mulac-Jericevic *et al.* 2003, Boonyaratanakornkit *et al.* 2008, Lanari *et al.* 2012), while PR-A has anti-proliferative properties (Saitoh *et al.* 2005, Boonyaratanakornkit *et al.* 2008, Khan *et al.* 2012). Although the ER and the PR are the best-known prognostic markers of breast cancer (Silvermana *et al.* 2011), other steroid receptors such as the androgen receptor (AR) as well as the glucocorticoid receptor (GR), have been implicated in the etiology and pathogenesis of hormone-dependent breast cancer (Teulings *et al.* 1980, Conzen 2008). In fact, current evidence in the literature suggests that both the AR and GR display anti-proliferative effects and may thus have protective roles in breast cancer (Isola 1993, Kuenen-Boumeester *et al.* 1996, Moinfar *et al.* 2003, Katalinic and Rawal 2008, Peters *et al.* 2009, Robinson *et al.* 2011).

It is noteworthy that some steroid receptors have been reported to be co-expressed in breast cancer tumours (Teulings *et al.* 1980, Ciocca *et al.* 2006, Gago *et al.* 2006, McNamara *et al.* 2013). For example, it is known that the AR can be co-expressed with the ER in breast cancer tumours (Kuenen-Boumeester *et al.* 1996, Moinfar *et al.* 2003, Castellano *et al.* 2010). Interestingly, the expression of the AR in ER-positive breast cancer tumours is associated with a more favourable prognosis (Moinfar *et al.* 2003, Alshenawy 2012, Qu *et al.* 2013). Since the AR is expressed in most tumours, and as current therapies targetting estrogen signalling pathways have been shown to be ineffective in some patients due to the development of drug resistance (Martin *et al.* 2005, Ciruelos *et al.* 2014), a drug targetting the AR signalling pathway, could potentially be beneficial. It is important to understand the AR signalling pathways so that they can be targetted for the development of improved breast cancer therapies (Brentani *et al.* 1986, Birrell *et al.* 1998, McNamara *et al.* 2013). Therefore the objective of this literature review is to highlight the respective roles of the ER subtypes and the AR in breast cancer biology, by focusing on the mechanism of action of these steroid receptors when bound to either cognate or synthetic ligands. The implications of crosstalk between these receptors for breast cancer biology will also be discussed.

1.2. Molecular mechanism of action of steroid receptors

The intracellular effects of steroid hormones are mediated via binding to members of the steroid receptor family, a subfamily of the nuclear receptor family, and includes the ER, PR, AR, GR and mineralocorticoid receptor (MR) (Gasc and Baulieu 1987, Couse and Korach 1999, Lu *et al.* 2006, Helsen and Frank 2014). These receptors are ligand-activated transcription factors with similarities in their structure and function.

1.2.1. General structure of steroid receptors

As shown in figure 1.1, steroid receptors consist of five distinct functional domains, designated A-E, with ERα having an additional, carboxy-terminal F domain (Sommer and Fuqua 2001,

Balfe *et al.* 2004, Yang *et al.* 2008, Charitidi *et al.* 2009, Jin and Li 2010). The amino-terminal A/B domain contains the variable ligand-independent activation function (AF)-1 domain, which is important for protein-protein interaction with transcription factors and cofactors. This is followed by a highly conserved and centrally located DNA binding domain (DBD) (C-domain), a hinge region (D-domain) and a moderately conserved ligand binding domain (LBD) (E-domain) situated at the carboxy-terminal (figure 1.1) (Jin and Li 2010, Forrest and Swaroop 2012); reviewed in (Bain *et al.* 2007); reviewed in (Africander *et al.* 2011)).

The DBD is highly homologous between the steroid receptors and comprises two zinc-finger motifs. The α-helix in the first zinc finger plays an important role in enabling the DBD to recognize specific palindromic DNA sequences known as steroid response elements (SREs) (Saji *et al.* 2005, Wu and Safe 2007, Charitidi *et al.* 2009, Helsen *et al.* 2012, McEwan 2012), while the second zinc finger is important for receptor homo- or heterodimerization (Shaffer and Gewirth 2002, Helsen and Frank 2014). The DBD is linked to the LBD via a short highly variable hinge region (D domain) (Wu and Safe 2007, Helsen *et al.* 2012, Hudson *et al.* 2014), which contains sites for post-translational modifications such as phosphorylation, methylation and sumoylation (Anbalagan *et al.* 2012, Helsen and Frank 2014). A second activation function domain (AF-2), is located in the LBD, and is necessary for recruiting various cofactors and for the induction of the transcriptional activity of the receptor (Kumar and Chambon 1988, Xu and Li 2003, Bain *et al.* 2007). It should be noted that the AF-2 transcriptional activity is ligand-dependent, while that of AF-1 is ligand-independent. Ligand-dependent recruitment of coregulators to the AF-2 domain occurs via a hydrophobic cleft in this domain, which is shielded by an amphipatic α-helix in the absence of ligand (Griekspoor *et al.* 2007). The presence of ligand repositions this helix, thereby allowing coregulator binding via conserved LXXLL motifs found in the AF-2 domain (Weatherman *et al.* 1999). The sequence homology among the LBDs of steroid receptors is moderately conserved, with about a 50-70% sequence homology between the AR, GR, MR and PR, while the LBDs of the ER and PR share only

about 28% sequence homology, and that of the GR and ER only about 30% (Fuller 1991, Moras and Gronemeyer 1998, Maeda 2001). Lastly, the F domain, unique to ERα, contains functional elements which are responsible for functions such as controlling the interaction of ERα with steroid receptor coactivator-1 (SRC-1), and plays an important role in the receptor's ability to distinguish between agonists and antagonists (Montano *et al.* 1995, Koide *et al.* 2007).

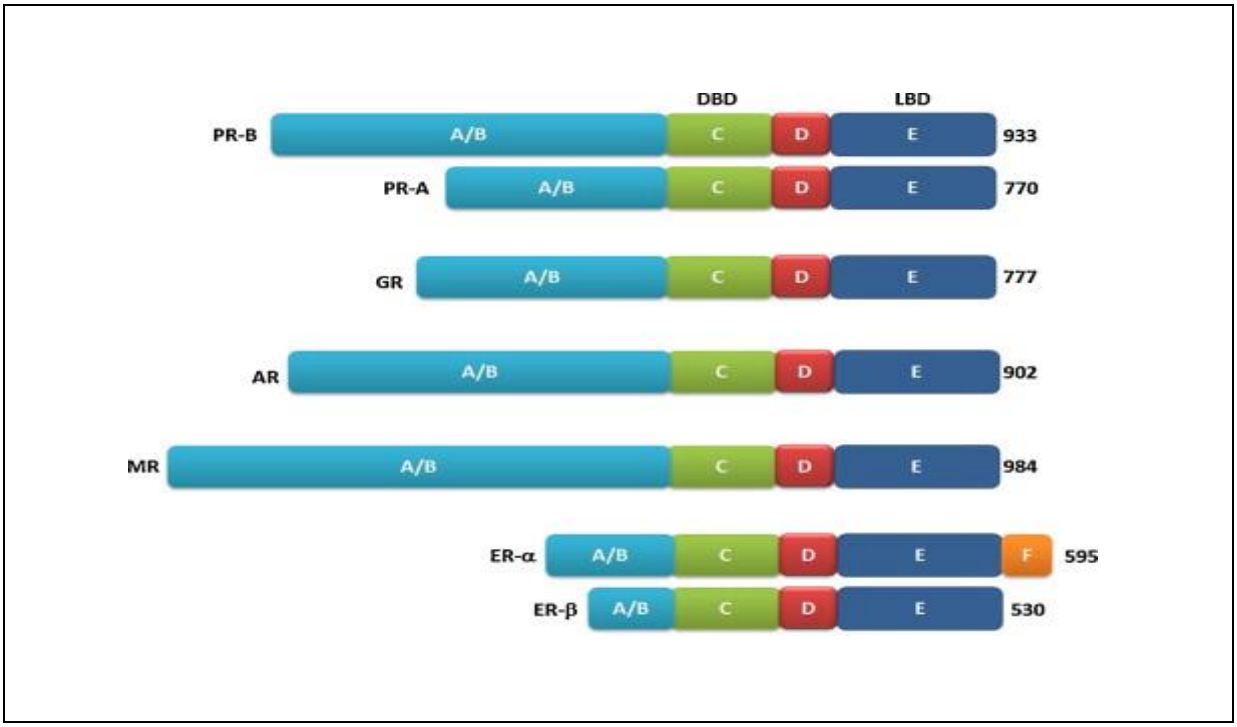

Figure 1.1. A schematic illustration of the general structure of steroid receptors. The A/B region denotes the variable N-terminal domain containing the activation function-1 (AF-1) transactivation domain, C is the conserved DNA binding domain (DBD), D denotes the hinge region, and E is the carboxy-terminal moderately conserved ligand binding domain (LBD) containing the AF-2 transactivation region. ERα, contains an additional F domain. The numbers represent the amino acid length of the steroid receptors. Figure taken from (Africander *et al.* 2011).

Most unliganded steroid receptors are found in the cytoplasm, however, the ER and PR are found mainly in the nucleus (Brosens *et al.* 2004). These unliganded steroid receptors are sequestered in an inactive form in a large molecular complex organised around the molecular chaperone heat shock proteins, Hsp90 and Hsp70 (Pratt and Toft 2003, Gougelet *et al.* 2007).

When activated by ligand, steroid receptors modulate gene expression via two mechanisms, namely transactivation and transrepression.

1.2.2. Transactivation

Ligand-dependent transcriptional activation of genes occurs when small lipophilic hormones diffuse across the plasma membrane and bind to the LBD of the steroid receptor, leading to a conformational change in the steroid receptor and translocation to the nucleus of those steroid receptors found predominantly in the cytoplasm (figure 1.2) (Zhao *et al.* 2010, Murphy 2011). Once in the nucleus, the hormone-bound steroid receptor binds as a dimer to specific SREs found in the promoters of steroid responsive target genes (Beato and Klug 2000, Kumar and Litwack 2009). For example, the ER binds to SREs called estrogen response elements (EREs), while the PR binds to progesterone response elements (PREs). The SREs for PR, AR, MR and GR are highly homologous and consist of the palindromic sequence 5'-GGTACAnnnTGTTCT-3' (Beato *et al.* 1989), while the ER recognizes the alternative 5'-GGTCAnnnTGACC-3' palindromic sequence (Klock *et al.* 1987, Klein-Hitpass *et al.* 1988, Brosens *et al.* 2004). The binding of a steroid receptor to the SRE, is followed by recruitment of coregulators, chromatin remodelling, leading to the recruitment of the basal transcriptional machinery (Beato and Klug 2000, Perissi and Rosenfeld 2005, Lu *et al.* 2006). This leads to the activation of gene transcription in a process known as transactivation (figure 1.2).

1.2.3. Transrepression

Steroid receptors can also negatively regulate transcription of genes by binding to DNA-bound transcription factors such as nuclear factor kappa-B (NFκB), activator protein-1 (AP-1) and CCAAT enhancer binding protein (C/EBP) (Ueda *et al.* 1994, Harrington *et al.* 2003, Pascual and Glass 2006, Frasor *et al.* 2009) in a process called transrepression (Harrington *et al.* 2003,

Lu *et al.* 2006, Carter and Stephen 2014). Although the ER, AR, PR and MR have all been shown to interact with NFκB to repress the expression of target genes (Kalkhoven *et al.* 1996, Palvimo *et al.* 1996, Ray *et al.* 1997, Aarnisalo *et al.* 1998, McKay and Cidlowski 1999, Kalaitzidis and Gilmore 2005, Sønder *et al.* 2006, Matter and Handschin 2007, Bergmann *et al.* 2010, Chantong *et al.* 2012, Drummond and Fuller 2012, Africander *et al.* 2014), the mechanism of transrepression is best studied for the GR through its interaction with NFkB and AP-1 (McKay and Cidlowski. 2000, Pascual and Glass 2006, Newton and Holden 2007, De Bosscher *et al.* 2008, Beck *et al.* 2009). NFκB is a transcription factor that is activated by many stimuli such as the tumor promoter, phorbol 12-myristate 13-acetate (PMA) (Lee *et al.* 2002, Chang *et al.* 2005) and proinflammatory cytokines such as tumour necrosis factor-alpha TNF-α (Scarpati and Sadler 1989, Ravi and Bedi 2004, Lee *et al.* 2007, Beck *et al.* 2009). Upon ligand binding to the GR, the liganded GR translocates to the nucleus where it tethers to DNA-bound NFκB, thereby inhibiting NFκB-driven gene expression (figure 1.2) (Ghosh *et al.* 1998, De Bosscher *et al.* 2008). Similar to the mechanism of transactivation, but less understood, transrepression also involves the binding of multiprotein complexes on DNA and the modulation of chromatin structure (Kassel and Herrlich 2007, Africander *et al.* 2011). Considering that all steroid receptors have essentially identical mechanisms of action for transactivation, it is probable that they repress genes by a similar mechanism to that of the GR. As this study focuses on the transcriptional activity of the ER and AR, a more detailed discussion on these receptors will follow.

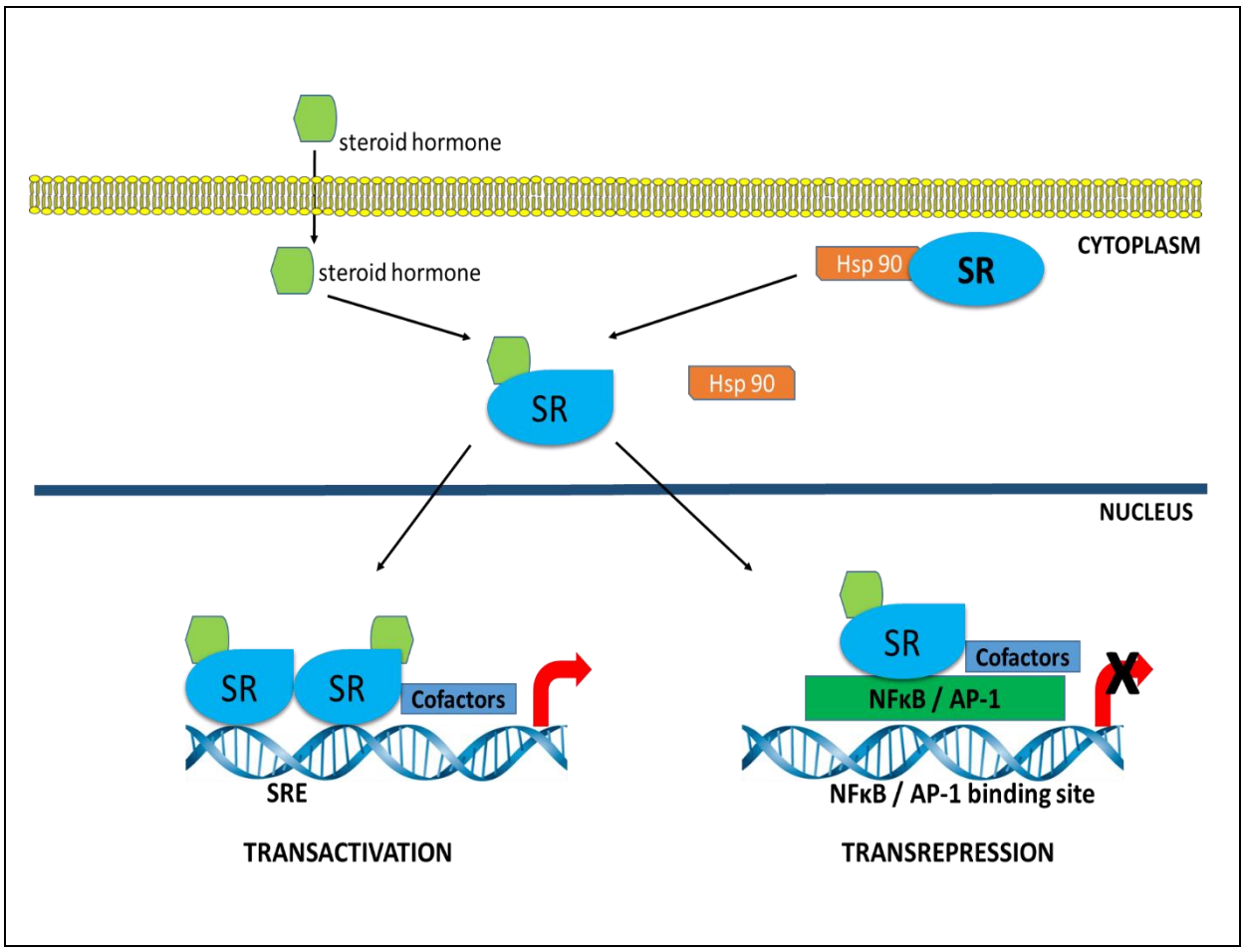

Figure 1.2. An illustration of the classical (general) mechanism of action of steroid receptors. SR: steroid receptor, SRE: steroid response element, Hsp90: heat shock protein 90, NFκB: nuclear factor kappa B, AP-1: activator protein. Co-regulators (represented by cofactors in the diagram), result in the opening of the chromatin structure and recruitment of basal transcription machinery. Redrawn from (Africander *et al.* 2011).

1.3. Molecular mechanisms of estrogen action

1.3.1. Production of estrogens and their physiological actions

Estrogens are female sex steroid hormones that play an important role in various tissues of the human body (Gougelet *et al.* 2005, Thomas and Potter 2013). Figure 1.3 shows the many examples of these roles, which include maintenance of bone density as well as the growth and proliferation of breast tissue (Gruber *et al.* 2002).

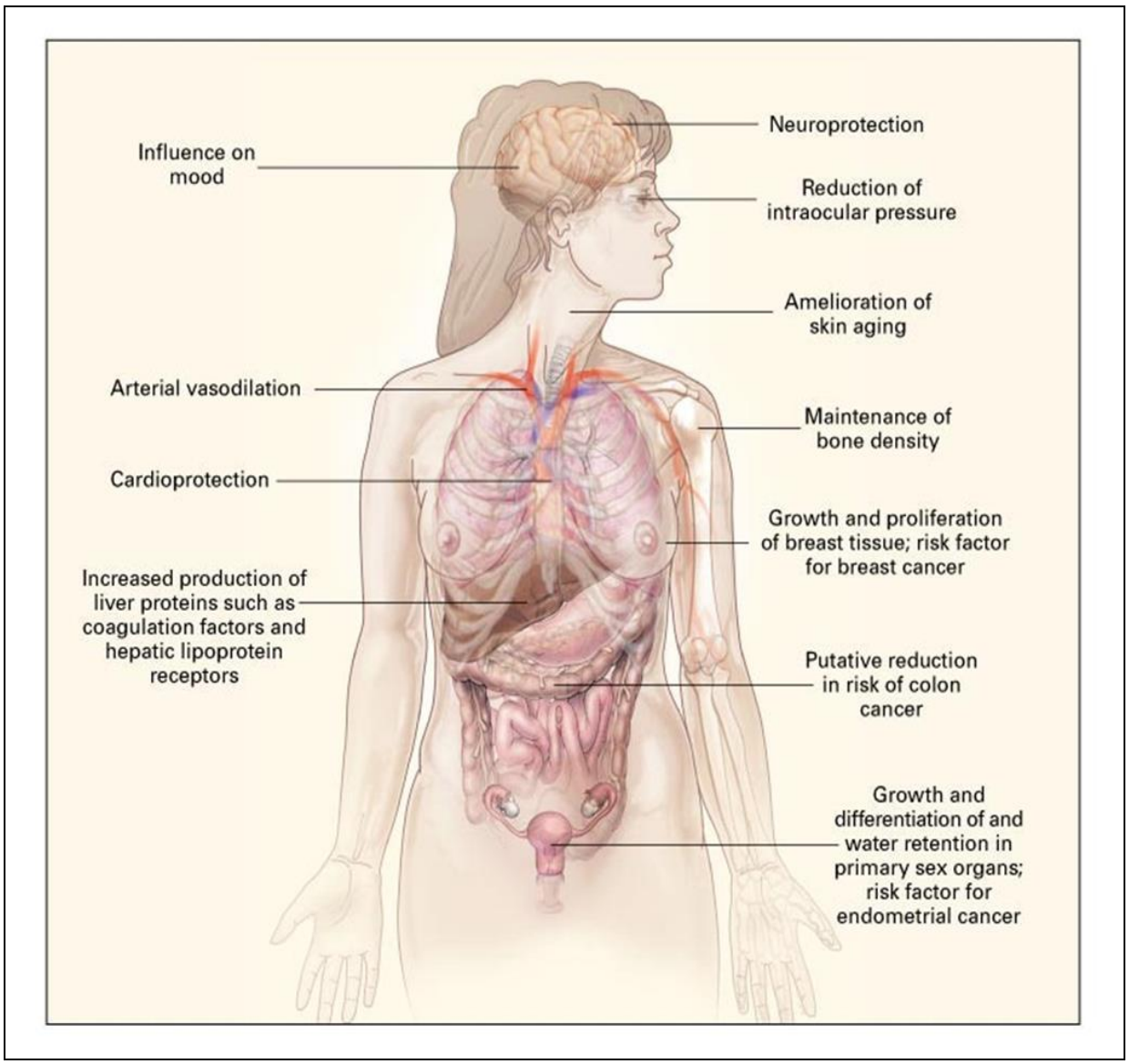

Figure 1.3. Estrogens act on multiple organ systems. The figure illustrates the diverse actions of estrogens on different organs in the human body. Figure taken from (Gruber *et al.* 2002).

Three types of estrogens are produced within the female body namely, estrone (E_1), 17β-estradiol (E_2), and estriol (E_3) (Thomas and Potter 2013, Samavat and Kurzer 2015). E_2 is mainly produced in the ovaries (Simpson 2002, Tsuchiya *et al.* 2005), while E_1 and E_3 are primarily produced in the liver from E_2 (Gruber *et al.* 2002, Tsuchiya *et al.* 2005). As shown in figure 1.4, E_2 is formed from testosterone either directly by the action of the aromatase (CYP19) enzyme, or indirectly through the reduction of E_1 by 17β-hydroxysteroid dehydrogenase (17β-HSD) isozymes. The 17β-HSD2 enzyme oxidises testosterone to androstenedione, which is

aromatized to E_1 (Tsuchiya *et al.* 2005, Knower *et al.* 2013, Thomas and Potter 2013, Samavat and Kurzer 2015). E_3 is synthesized via the action of the enzyme 17β-HSD1 on the intermediate 16α-hydroxyestrone, which is produced from E_1 via cytochrome P450 3A4/5 (CYP3A4 or CYP3A5) (Huang *et al.* 1998). E_1 is the inactive form of E_2 (Andersson and Moghrabi 1997, Abplanalp *et al.* 1999) and has been shown to inhibit cell division of the MCF-7 breast cancer cell line (Jozan *et al.* 1979). Although previous studies have suggested that high levels of E_3 protect women against breast cancer (Lemon 1975, Wotiz *et al.* 1978, Lemon *et al.* 1989), some studies have shown that E_3 can increase the growth of breast cancer cells and activate expression of ERE-containing genes to the same extent as E_2 (Katzenellenbogen 1984, Pang and Faber 2001, Diller *et al.* 2014). As E_2 is the most biologically active estrogen, with circulating levels in the high picomolar to nanomolar range in the reproductive phase of women (Chatterton *et al.* 2003, Watson *et al.* 2008, Thomas and Potter 2013, Samavat and Kurzer 2015), we will only focus on the actions of E_2.

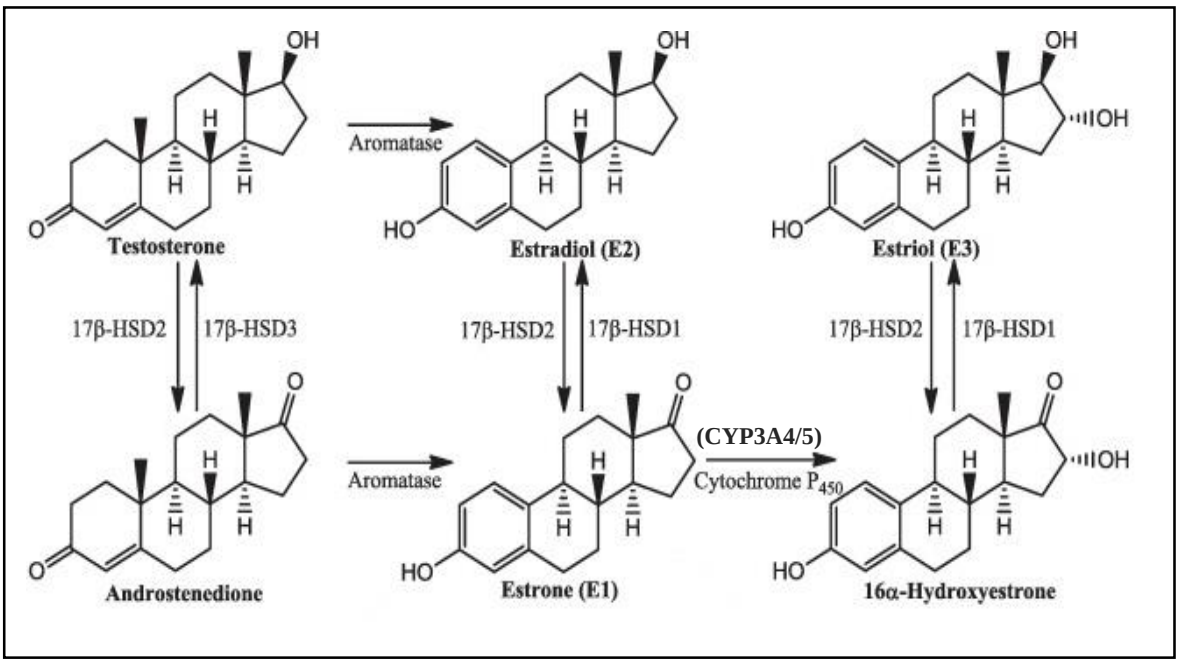

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Figure 1.4. An illustration of the estrogen metabolism pathway. Testosterone is the main precursor for estrogen production, which involves different cytochrome P450 enzymes. Aromatase is the major enzyme involved in the production of E_2 and E_1 from the androgen precursors (testosterone and androstenedione), 17β-HSD plays a role in the production of E_1 and E_3, while CYP3A4/5 is involved in the production of E_3 from E_1. Adapted from (Thomas and Potter 2013).

E_2 is released into the bloodstream after production and ~37% binds to sex hormone binding globulin (SHBG) and ~60% binds to albumin for transport to target tissues (Dunn *et al.* 1981), while only ~3% is left free or unbound (Siiteri 1987, Pardridge 1988, Petra 1991, Gruber *et al.* 2002). Free E_2 diffuses into target tissues where its biological actions are primarily mediated by binding to estrogen receptors (Matthews and Gustafsson 2003, Balfe *et al.* 2004, Faulds *et al.* 2012), and these actions include the normal female reproductive processes as well as playing a role in the regulation of a number of diseases. Examples of the latter include osteoporosis, coronary heart disease (CHD), Alzheimer's disease and hormone-dependent cancers, which include endometrial, ovarian, prostate and breast cancer (Ingemar 2000, Paranjape *et al.* 2005, Li-Min *et al.* 2012, Wen-Yang *et al.* 2012, Brown and Hankinson 2014, Li *et al.* 2014, Hamed and Mindy 2015). It is important to note that the role of E_2 in the above-mentioned processes can be either positive or negative. Examples of positive effects include reports that high levels of E_2 prevents the development of Alzheimer's disease (Giacobini 1998, Xing *et al.* 2013, Li *et al.* 2014), while the use of E_2 plus progestin in HRT has been associated with a decreased risk of osteoporosis (Lee *et al.* 2013, Nelson *et al.* 2013) and coronary heart disease (CHD) (Grodstein *et al.* 1996, Paranjape *et al.* 2005, Hodis and Mack 2014). In contrast to these positive effects, E_2 has been shown to increase the risk of endometrial and ovarian cancer via several mechanisms such as cell proliferation, decreased apoptosis and increased DNA damage (Henderson and Feigelson 2000, Cunat *et al.* 2004, Liang *et al.* 2009, Zhang *et al.* 2012, Brown and Hankinson 2014, Ribeiro and Freiman 2014). Similarly, long-term exposure to E_2 has been shown to be linked to an increased risk of breast cancer and may lead to tumor growth via

mechanisms such as increased DNA damage and cell proliferation (Fox *et al.* 2008, Santen *et al.* 2009, Yue *et al.* 2013).

1.3.2. Diverse molecular pathways are involved in ER-mediated estrogen actions

1.3.2.1. ER subtypes

The main actions of estrogen are genomic and are mediated by the ER subtypes. The two main functional ER subtypes, ERα and ERβ, are transcribed from different genes (Menasce *et al.* 1993, Matthews and Gustafsson 2003). ERα is a 66 kDa protein, which was discovered in the late 1960s (Toft *et al.* 1967) and was isolated and purified from the human MCF-7 breast cancer cell line in the late 1980s (Walter *et al.* 1985, Green *et al.* 1986). It is expressed in the female reproductive system (breast, ovary, uterus), male reproductive system (testis, epididymis, prostate gland) and various other tissues such as the pituitary gland, kidney, bladder, liver, heart, thymus and adrenal gland (Ogawa *et al.* 1998, Pearce and Jordan 2004). ERβ is a 56 kDa protein, which was only identified in 1996 (Kuiper *et al.* 1996, Mosselman *et al.* 1996, Katzenellenbogen and Korach 1997, Enmark and Gustafsson 1999), and like ERα, ERβ is expressed in the female and male reproductive systems as well as various other tissues such as the bladder, lung, adrenal and heart (Iwao *et al.* 2000, Roger *et al.* 2001, Farquhar *et al.* 2009).

Human ERα and ERβ have similar structural domains (figure 1.5) and differ mainly in their amino acid length (Nilsson *et al.* 2001, Pearce and Jordan 2004, Ascenzi *et al.* 2006). The N-terminal domain of the ERs has only about 30% identity, with ERβ being reported to lack AF-1 activity (Pearce and Jordan 2004). The DBD of the two ERs has 96% identity, suggesting that both receptors would bind to similar sites in target genes. The hinge region, like the N-terminal domain, is not highly conserved between the ERs (30% homology). The LBD's of the two subtypes have a 53% homology, and show slight differences in ligand binding specificity

(Mosselman *et al.* 1996, Pearce and Jordan 2004). For example, E_2 has been shown to have a slightly higher binding affinity for ERα as compared to ERβ, while phytoestrogens such as genistein has a higher binding affinity for ERβ (Verhoog *et al.* 2007).

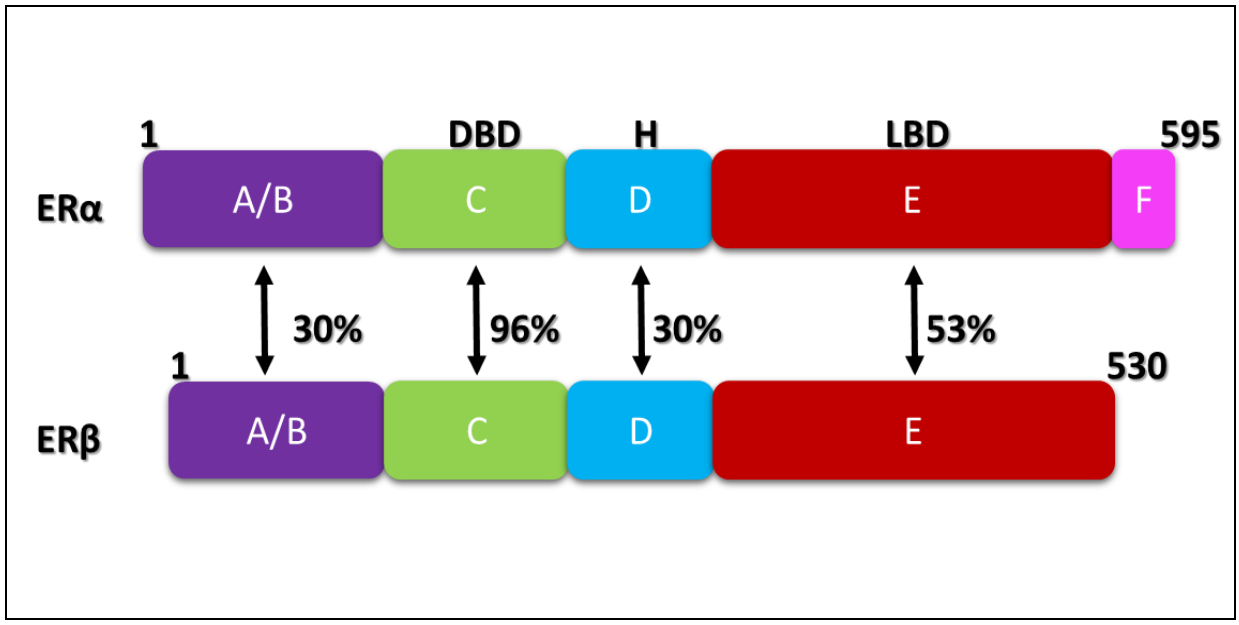

Figure 1.5. Structural homology between human ERα and ERβ. The various domains are represented by the letters A-F, with the amino acid sequence homology between the individual domains shown as a percentage. The A/B region denotes the variable N-terminal domain containing the AF-1 domain, C is the DBD, D denotes the hinge region which connects the DBD to the LBD, E is the LBD, which contains the AF-2 domain. ERα, contains an additional F domain. The numbers represent the amino acid length of the steroid receptors. Figure redrawn from (Pearce and Jordan 2004, Africander *et al.* 2011).

1.3.2.2. Molecular pathways

ERs can regulate biological processes through several distinct signaling pathways, which include both ligand-dependent and ligand-independent mechanisms (figure 1.6). The classical genomic mechanism of steroid receptor action is ligand-dependent (illustrated in figure 1.2), and for the ER, involves E_2 binding to the ER, and the subsequent direct binding of the E_2-bound ER to an ERE (figure 1.6A) or tethering to another transcription factor (figure 1.6B) (Gruber *et al.* 2002, Simoncini *et al.* 2004, Yager and Davidson 2006, Heldring *et al.* 2007). These nuclear actions lead to increased gene expression of target genes via the ERE, and

decreased transcription of target genes via tethering to DNA-bound transcription factors such as NFκB and AP-1 (Stein and Yang 1995, Galien and Garcia 1997, Yamei *et al.* 2007, Robertson *et al.* 2010). Interestingly, the ER has been shown to repress NFκB signalling at various levels of transcriptional regulation (Kalaitzidis and Gilmore 2005, Drummond and Fuller 2012). For example, the ER can repress NFκB signaling by (i) suppressing inhibitor of kappa B (IκB) kinase (IKK) activity, (ii) preventing the degradation of IκB proteins, (iii) blocking NFκB from binding to DNA and (iv) competing with NFκB for coactivators (Kalaitzidis and Gilmore 2005, Drummond and Fuller 2012). However, there is limited information in the literature regarding ER subtype-specific-mediated mechanisms of transrepression. At least one study has shown that E_2 acting via both ER subtypes, can repress the expression of an NFκB containing promoter-reporter construct (Visser *et al.* 2013), while one other study showed that E_2 acting via ERα was able to cause repression of three TNF-α-induced proinflammatory genes (Cvoro *et al.* 2011).

Although the classical mechanism of action can take hours to achieve maximal E_2-induced gene responses (Gruber *et al.* 2002, Simoncini *et al.* 2004, Heldring *et al.* 2007), some of the ligand-activated ER responses are more rapid (Watsona *et al.* 1999, Gruber *et al.* 2002, Wong *et al.* 2002, Simoncini *et al.* 2004, Song *et al.* 2005, Song and Santen 2006). An example of the latter is the non-genomic mechanism involving membrane-bound ER (figure 1.6C) and most likely involves other membrane bound receptors such as G-protein-coupled receptors (GPRCs). In this mechanism the ligand activates the membrane-associated ER, leading to the initiation of signaling cascades via second messengers (SM). These SM affect ion channels or increase nitric oxide (NO) levels in the cytoplasm, thereby causing rapid physiological responses without involving gene regulation (Hall *et al.* 2001, Stefan *et al.* 2001, Björnström and Sjöberg 2005, Heldring *et al.* 2007, Silva *et al.* 2010, Ozgyin *et al.* 2015). Lastly, growth factor (GF) signalling (figure 1.6D) can lead to the activation of protein kinase cascades, which result in the

phosphorylation of ERs and the subsequent binding to EREs, thus modulating gene regulation via a ligand-independent mechanism (Björnström and Sjöberg 2005, Heldring *et al.* 2007).

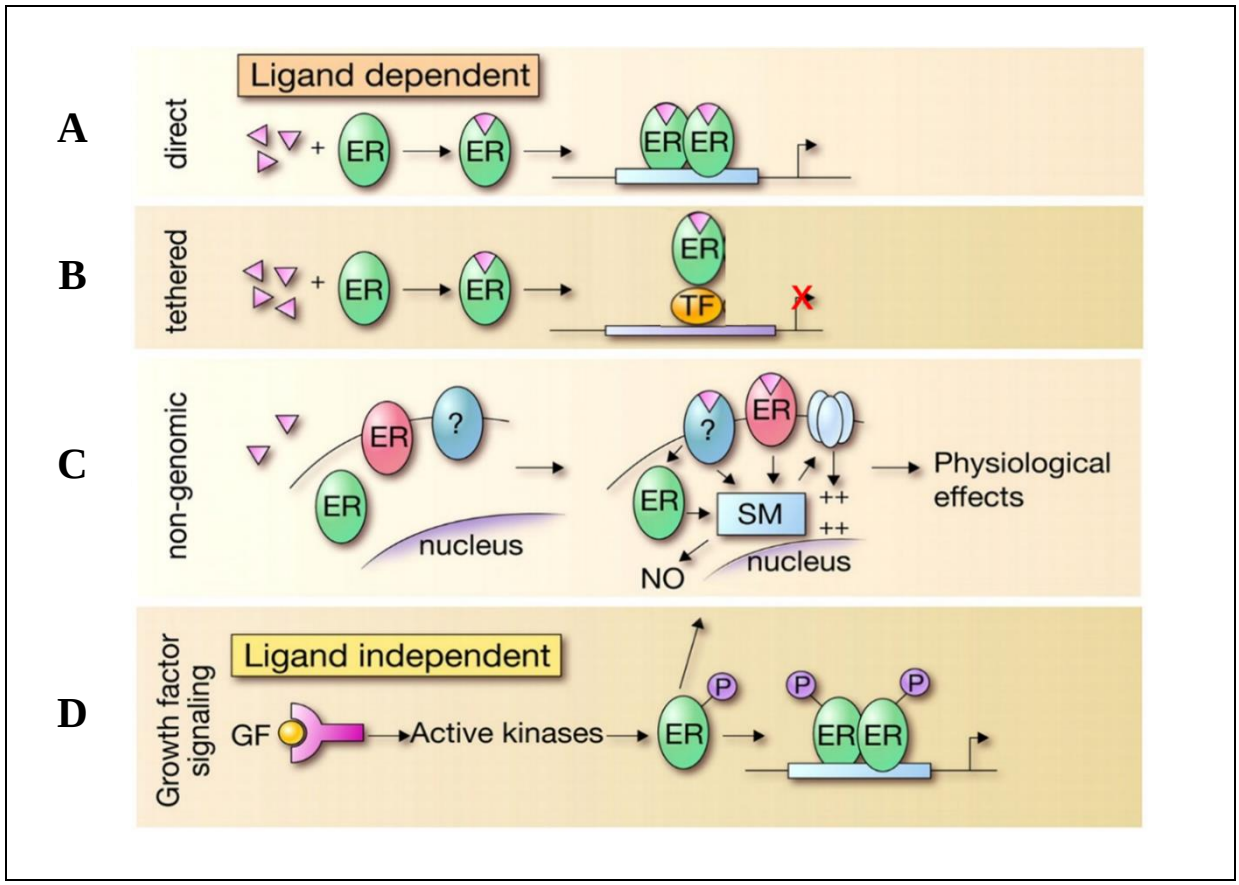

Figure 1.6. Molecular pathways used in the regulatory actions of ERs. The classical (direct) pathway (A) involves ligand-bound ER binding directly to EREs leading to increased transcription of target genes. Alternatively, the tethered pathway (B) involves indirect binding to DNA via protein-protein interactions with other transcription factors resulting in decreased transcription of target genes. The non-genomic pathway (C) involves ligand activation of membrane receptors, which leads to the initiation of signaling cascades via second messengers (SM), increasing nitric oxide (NO) levels and ultimately leading to altered physiological responses. Lastly the ligand-independent mechanism (D) involves activation via growth factor (GF) signalling leading to the activation of kinases that phosphorylate the ERs thereby activating them and modulating gene transcription. Figure modified from (Heldring *et al.* 2007).

1.4. The role of E_2 and ER subtypes in breast cancer biology

1.4.1. E_2 and ERα

Numerous clinical and epidemiological studies have indicated that exposure to E_2 is associated with increased risk of developing breast cancer (Clemons and Goss 2001, Yager and Davidson 2006). The cellular mechanisms by which E_2 increases breast cancer risk is an area of ongoing study but at least two possibilities have been suggested (figure 1.7) (Miller 2004). Firstly, E_2 binds to the ER causing altered gene expression, which results in uncontrolled or excessive cell proliferation (figure 1.7A). Interestingly, the E_2-induced proliferative effects on breast cancer cells in humans can be either direct or indirect (Clemons and Goss 2001). The direct E_2-induced mechanism of cell proliferation is initiated by the induction of enzymes and proteins involved in nucleic acid synthesis and activation of specific oncogenes (Clemons and Goss 2001), while the secretion of hormones such as prolactin and the production of GFs such as transforming growth factor alpha (TGFα) initiates the indirect mechanism of cell proliferation (Clemons and Goss 2001). The second mechanism whereby it has been suggested that E_2 causes increased risk of breast cancer comes from evidence suggesting that the metabolism of E_2 to genotoxic agents causes direct DNA mutations (figure 1.7B) (Yager and Leihr 1996, Miller 2004, Yager and Davidson 2006). E_2 is metabolized via catechols (organic compounds), particularly 2- or 4- hydroxyestradiol or hydroxyestrone, to produce quinones. The latter interacts with and mutates DNA, initiating carcinogenesis (figure 1.7B) (Miller 2004, Yager and Davidson 2006). Considering that this thesis focusses on some parts of the pathway shown in figure 1.7A, and that the ERα subtype has predominantly been implicated in the development and progression of breast cancer, only the role of E_2 via the ERα will be further discussed.

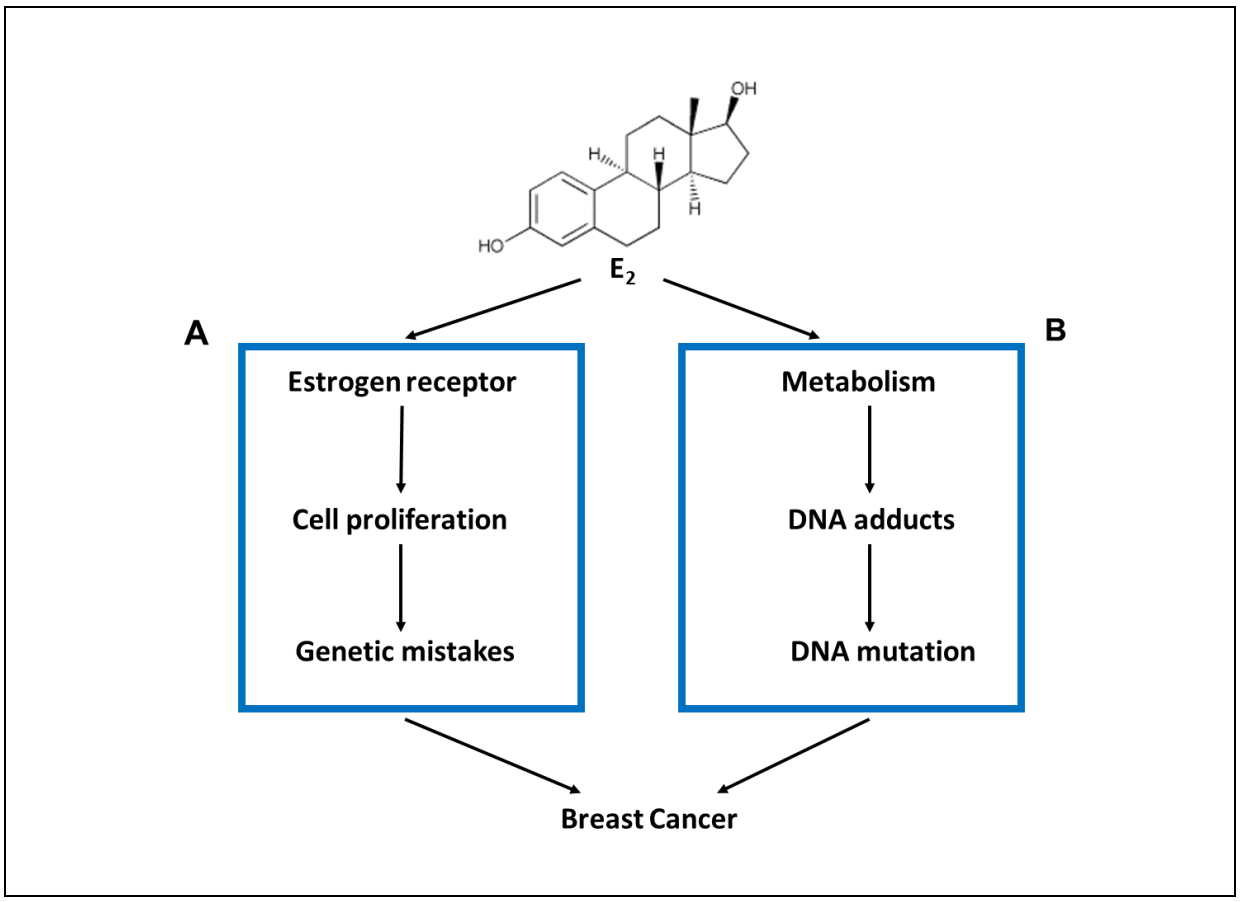

Figure 1.7. Two pathways indicating the mechanisms of E_2 carcinogenesis. The diagram illustrates two mechanisms that contribute to the initiation of breast cancer. (A) ER-mediated increase in cell proliferation and (B) metabolism of E_2 to genotoxic agents such as quinones, causing DNA mutation. Redrawn from (Miller 2004).

ERα is expressed in almost 70% of breast cancers (Jensen and Jordan 2003, Pearce and Jordan 2004, Conzen 2008). Both *in vivo* and *in vitro* studies have reported that ERα is crucial for the promotion of breast cancer cell growth and survival by E_2 (Cunha *et al.* 1997, Couse and Korach 1999, Frasor *et al.* 2003, Hartman *et al.* 2009, Brisken and O'Malley 2010, Powell *et al.* 2012, Huang *et al.* 2014b). For example, despite the presence of high levels of E_2, no breast cancer tumours were detected in ERα knock-out mice (Couse and Korach 1999). Current therapies for E_2-dependent breast cancer are thus directed towards either (i) preventing the interaction of E_2 with the ERα using selective ER modulators (SERMs) or selective estrogen receptor downregulators (SERDs) (Jordan 2007, Patani and Martin 2014) or (ii) by inhibiting the synthesis of E_2 using aromatase inhibitors (AI's) (Mirzaie *et al.* 2013, Nantasenamat *et al.* 2013,

Akçay and Bayrak 2014). The latter includes compounds such as letrozole and anastrozole, which bind reversibly to the aromatase enzyme, thereby decreasing the levels of E_2 available to promote tumour formation or progression (Kijima *et al.* 2005, Gil *et al.* 2013). Studies have shown that anastrozole decreases cell proliferation in breast cancer cell lines (Miller and Jackson 2003, Kijima et al. 2005, Gil et al. 2013) and reduces tumour size when administered to breast cancer patients (Miller and Jackson 2003, Kijima *et al.* 2005, Gil *et al.* 2013). SERMs on the other hand are ligands that bind the ERα and act as either agonists or antagonists depending on the type of tissue (Howell *et al.* 2004, Renoir 2012). In the breast however, SERMs act as ERα antagonists (Macgregor and Jordan 1998, O'Regan and Jordan 2002) and include tamoxifen, the most frequently used drug in first-line clinical therapy for the treatment and prevention of hormone-sensitive breast cancer (Ogawa *et al.* 1998, Girault *et al.* 2006, Zervoudis *et al.* 2014, Lim *et al.* 2015), as well as raloxifene (Kijima *et al.* 2005, Zhang *et al.* 2013). Both these ligands have been shown to inhibit breast cancer cell growth (Speirs *et al.* 1999, Fowler *et al.* 2004, Howell *et al.* 2004, Speirs *et al.* 2004, Kijima *et al.* 2005, Lange 2008) by antagonizing E_2-induced gene expression and proliferation. SERDs are pure antagonists that do not have any agonist activity via the ERα in any tissue (Macgregor and Jordan 1998, O'Regan and Jordan 2002, Howell *et al.* 2004, Renoir 2012), and ICI 182,780, also known as fulvestrant or faslodex, and RU58668 (Pearce and Jordan 2004, Renoir 2012, Ciruelos *et al.* 2014). Clinically, faslodex is used as second-line treatment when patients fail to respond to tamoxifen (Lynn 2004, Martin *et al.* 2005, Howell 2006, Ciruelos *et al.* 2014). SERDs inhibit ERα-mediated transcriptional activity by down-regulating ERα levels, as these ligands target the ERα for proteasome-dependent degradation (Alarid *et al.* 1999, Lonard *et al.* 2000, Meiyun *et al.* 2003, Howell 2006, Renoir 2012, Ciruelos *et al.* 2014).

Several studies have shown that E_2-bound ERα promotes the development and progression of breast cancer by upregulating the expression of genes, such as c-myc, c-fos and cyclin D1, that play a role in the cell cycle and induce breast cancer cell (Helguero *et al.* 2005, Hartman *et al.*

2009, Brisken and O'Malley 2010), and/or by eliciting anti-apoptotic effects through the upregulation of the anti-apoptotic protein Bcl-x and downregulation of the pro-apoptotic protein Bax (Helguero *et al.* 2005). Not only does E_2 stimulate breast cell proliferation and inhibit apoptosis via ERα, but it also plays a role in cell invasion and metastasis (Clarke *et al.* 2003, Jiang *et al.* 2007). For example, E_2 stimulates the invasive and metastatic potential of the ERα-positive MCF-7 breast cancer cell line (Shafie and Liotta 1980, Osborne *et al.* 1985, Cos *et al.* 1998).

1.4.2. ERβ and its interplay with ERα

Immunohistochemical analysis has shown that both ERα and ERβ are expressed in the normal breast, and that ERβ expression is more abundant than ERα (Clarke *et al.* 1997, Russo *et al.* 1999, Speirs *et al.* 2004). However, more ERα than ERβ is expressed during the development of breast cancer (Leygue *et al.* 1998, Roger *et al.* 2001, Murphy *et al.* 2003, Shaaban *et al.* 2003). Interestingly, a small number of breast cancers express only ERα (~14%) or only ERβ (~18%) (Murphy *et al.* 2003, Skliris *et al.* 2008, Leung *et al.* 2012). It is important to note that the current evidence in the literature suggests that co-expression of ERα and ERβ in breast cancer patients is linked to a better prognosis than when ERα or ERβ alone is expressed (Omoto *et al.* 2001, Roger *et al.* 2001, Paruthiyil *et al.* 2004, Lin *et al.* 2007). This is due to the ability of ERβ to inhibit ERα-mediated cell proliferation as well as its ability to induce apoptosis (Leygue *et al.* 1998, Dotzlaw *et al.* 1999, Speirs *et al.* 1999, Zhao *et al.* 2008). For example, evidence from *in vitro* studies indicate that co-expression of ERβ in ERα-positive breast cancer cells inhibits the proliferative effects of E_2 via ERα (Williams *et al.* 2007, Hartman *et al.* 2009). In fact, even in the absence of E_2, co-expression of ERβ with ERα in the same cell line, promotes anti-proliferative and anti-tumour effects (Paruthiyil *et al.* 2004, Lin *et al.* 2007). It has been proposed that ERβ exhibits these effects by decreasing the expression of the E_2-responsive pro-proliferative (c-myc, cyclin A and cyclin D1) (Paruthiyil *et al.* 2004) and anti-apoptotic (Bcl-2

and Bcl-x) (Nilsen *et al.* 2000, Song and Santen 2003) genes, and/or increasing expression of anti-proliferative genes such as $p21^{Cip1}$ and $p27^{Kip1}$ (Paruthiyil *et al.* 2004, Lin *et al.* 2007). In contrast to ERα, the role of ERβ in breast cancer cell invasion is not well-studied, but the evidence from at least two studies suggest that ERβ inhibits breast cancer cell invasion, both *in vivo* and *in vitro* (Lazennec *et al.* 2001, Thomas *et al.* 2012). Furthermore, studies have shown that ERβ can antagonise ERα-mediated effects by either interfering with the recruitment of co-activators required for ERα-mediated actions, or by forming a heterodimer with ERα and thereby affecting the recruitment of this receptor to the promoters of E_2-responsive genes such as the PR (Fox *et al.* 2008).

The presence of ERβ in ERα-negative breast cancers is, however, not considered favourable. Studies indicate that in the absence of ERα, ERβ promotes proliferation by increasing the expression levels of the proliferative markers Ki67 (O'Neill *et al.* 2004, Skliris *et al.* 2006) and cyclin A (Fox *et al.* 2008). Although the molecular mechanism underlying this phenomenon is unclear, at least one study in the literature suggest that the phosphorylation status of ERβ may play a role (Tremblay *et al.* 1999). These authors showed that the phosphorylation of residues within the AF-1 domain in ERβ, due to an increase in mitogen-activated protein kinases (MAPK) activity, enhances the recruitment of the co-activator SRC-1, and thereby leads to enhanced transcriptional activity of ERβ (Tremblay *et al.* 1999).

1.5. Role of androgens and the AR in breast cancer biology

1.5.1. Androgens

For many decades, E_2 and the ERs were thought to be the main etiological factors implicated in breast cancer pathogenesis. However, recent evidence suggests that androgens and the AR are not only role players in prostate cancer (Weller *et al.* 2014, Karantanos *et al.* 2015), but may also play a role in the development and progression of breast cancer (Nicolás *et al.* 2007, Hu *et*

al. 2011, Robinson *et al.* 2011, Kotsopoulos and Narod 2012, Li *et al.* 2013). The reported function of androgens in breast cancer is, however, contradictory, as at least one study shows stimulatory effects (Birrell *et al.* 1995), while a number of other studies indicate inhibitory effects on the growth of breast cancer cells (Hackenberg *et al.* 1991, Birrell *et al.* 1998, Greeve *et al.* 2004). Stimulatory effects are supported by studies showing that the most potent natural androgen, 5α-dihydrotestosterone (DHT), has pro-proliferative effects in MCF-7 and MDA-MB-453 breast cancer cell lines (Birrell *et al.* 1995, Lin *et al.* 2009), suggesting an increased risk of breast cancer. In contrast, the apoptotic and anti-proliferative effects of the potent synthetic androgen, mibelorone (MIB) in T47-D and ZR-75-1 cells, may provide a mechanism to support its reported protective role in breast cancer pathogenesis (Nicolás Díaz-Chico *et al.* 2007, Dimitrakakis 2011, Kotsopoulos and Narod 2012, Coss *et al.* 2014, Lakis *et al.* 2014). Whether androgens elicit anti-proliferative or proliferative effects in cell lines appear to be dependent on various factors including, the specific cell line and androgen used, the concentration of the androgen used, as well as the absence or presence of E_2 (Birrell *et al.* 1995, Birrell *et al.* 1998).

In addition to the above-mentioned *in vitro* studies, both *in vivo* and clinical studies (Dauvois and Labrie 1989, Dauvois *et al.* 1989, Lin *et al.* 2009, Dimitrakakis 2011) have also shown that androgens can either inhibit or stimulate the growth of tumours in breast cancer patients (Sourla *et al.* 1998, Dimitrakakis *et al.* 2003). Clinical evidence suggests that the subcutaneous administration of testosterone in postmenopausal women may be beneficial as it reduces the incidence of breast cancer (Bitzer *et al.* 2008, Dimitrakakis and Bondy 2009, Glaser and Dimitrakakis 2013). The clinical use of testosterone, however, is also associated with negative effects. This is due to the aromatization of testosterone to E_2, thereby possibly increasing the risk of breast cancer (Cauley *et al.* 1999, Somboonporn and Davis 2004). Studies have suggested that DHT, which unlike testosterone cannot be aromatized to E_2, may reduce tumour growth. However, this effect depends on factors such as androgen concentration and cell line

used. Although some studies suggest that DHT may provide a new treatment option for breast cancer patients as it cannot be aromatized to E_2 (Ando *et al.* 2002, Chottanapund *et al.* 2013), DHT may have masculinizing side-effects and thus newly-developed selective androgen receptor modulators (SARMs) may be a better treatment option (Gao and Dalton 2007, Bhasin and Jasuja 2009).

1.5.2. Androgen receptor (AR)

At the molecular level, androgens mediate their effects via the AR (Birrell *et al.* 1998, Kandouz *et al.* 1999, Birrell *et al.* 2007). This intracellular steroid receptor is predominantly cytoplasmic in its ligand-free state and upon androgen binding, the AR translocates to the nucleus where, like the ER, it either positively or negatively regulates target gene expression (Gobinet *et al.* 2002, Lee and Chang 2003). Some AR regulated genes such as the prostate homeobox (NKX3) and kallikreins (KLK5 and KLK3, also known as prostate-specific antigen (PSA)), are associated with breast cancer pathogenesis. For example *in vivo* studies have shown that NKX3 inhibits ER signalling and therefore can be a potential regulator of hormone response in breast cancer (Corthals *et al.* 2000, Xu *et al.* 2001, Nelson *et al.* 2002, Holmes *et al.* 2008, Wang *et al.* 2009, Asch-Kendrick *et al.* 2014). Although PSA is generally regarded as a prostate cancer-specific gene, heightened PSA levels have also been observed in malignant breast cancers. The physiological relevance of the latter is not yet clear, although it has been suggested that PSA promotes breast cancer tumour progression and metastasis (Yu *et al.* 1998, Black *et al.* 2000).

As mentioned earlier, a number of studies have suggested that the AR, which is expressed in approximately 70-90% of primary breast tumours (Søreide *et al.* 1992, Garay and Park 2012) and in 25% of advanced breast tumours (Søreide *et al.* 1992, Birrell *et al.* 1998, Garay and Park 2012, Chottanapund *et al.* 2013), may play a critical role in breast cancer biology (Hu *et al.* 2011, Robinson *et al.* 2011, Li *et al.* 2013). It is proposed that the expression of the AR in breast

tumours inhibits tumour growth and metastasis of cancer, and as a result reduces the number of breast cancer deaths (Søreide *et al.* 1992, Birrell *et al.* 1995, Kulshreshtha *et al.* 2010, Chanplakorn *et al.* 2011, Loibl *et al.* 2011, Yu *et al.* 2011, McNamara *et al.* 2014b). Considering that the majority of breast cancers express the AR, and taking into account that the above-mentioned studies suggest that the AR inhibits breast cancer progression, it has been proposed that the current clinical classification of breast cancers, which focusses only on ERα, PR and human epidermal growth factor receptor 2 (HER2) status, should be altered to include the AR (Farmer *et al.* 2005, McNamara *et al.* 2014b). For example, basal (ERα-negative, AR-negative); luminal (ERα-positive, AR-positive); molecular apocrine (ERα-negative, AR-positive) (Farmer *et al.* 2005). The latter subtype is often referred to as triple negative breast cancer (TNBC) due to the lack of ERα, PR and HER2 expression (Nicholas *et al.* 2003, Anestis *et al.* 2015).

Luminal cancer is subdivided into luminal A (low Ki67 index, slow tumour growth) and B (high Ki67 index, high tumour growth) (Yanagawa *et al.* 2012), with the AR more abundantly expressed in luminal A tumours (Park *et al.* 2010, Loibl *et al.* 2011, Tsang *et al.* 2014). The co-expression of AR with ER is associated with smaller tumour size and no signs of relapse during endocrine treatment and chemotherapy (Castellano *et al.* 2010, Hu *et al.* 2011, Park *et al.* 2011, Tsang *et al.* 2014). The protective effect of the AR is attributed to its ability to inhibit the activity of ERα in patients with luminal breast tumours (Peters *et al.* 2009, Castellano *et al.* 2010). The role of the AR in TNBC is controversial. A study has shown that the expression of the AR in TNBC is associated with an increase in lymph node metastases and number of breast cancer deaths (Hu *et al.* 2011). This is thought to be due to the AR acting as a surrogate ERα and activating transcription of ERα-regulated genes thereby promoting tumour progression in patients (Robinson *et al.* 2011). However, other studies indicate that the presence of the AR in TNBC results in a reduced proportion of lymph node metastases and a better prognosis (Sutton *et al.* 2012, Tang *et al.* 2012). Furthermore, at least one study has shown that AR expression has no influence on TNBC (Mrklić *et al.* 2013).

In conclusion, although studies in the literature on the effects of androgens and the AR on breast cancer are contradictory, the AR is currently being considered as a therapeutic target for the treatment of breast cancer (Chottanapund *et al.* 2013). In ERα-positive (luminal) breast cancers, targetting AR activation may be ideal as the unliganded- or ligand-bound AR inhibits the transcriptional activity of ERα (Peters *et al.* 2009, Fioretti *et al.* 2014). However, in ERα-negative (molecular apocrine/TNBC) breast cancers, it may be beneficial to inhibit the actions of the AR as it may function as a surrogate ERα in the absence of the latter (Fioretti *et al.* 2014). From the above, it is clear that the function of the AR in breast cancer is determined by whether or not the ER is present. In the next section, we will thus discuss the current knowledge in the literature on the association between the ER subtypes and the AR in breast cancer.

1.5.3. Crosstalk between the ER subtypes and the AR

A number of clinical studies have showed that AR expression in ER-positive breast tumours is associated with lower grade disease, reduced invasiveness and longer disease-free survival (Castellano *et al.* 2010, Hu *et al.* 2011, Loibl *et al.* 2011, Park *et al.* 2011, Yu *et al.* 2011, Park *et al.* 2012, Qu *et al.* 2013, Vera-Badillo *et al.* 2013, Witzel *et al.* 2013, Tsang *et al.* 2014). However, these studies do not distinguish between the two ER subtypes expressed in these breast tumours. To the best of our knowledge, the few *in vitro* studies that have been conducted to gain insight into the interplay between the ER subtypes and AR in breast cancer mainly focused on the ERα subtype.

Figure 1.8 shows the proposed mechanisms by which the AR can either promote or inhibit breast cancer pathogenesis. In ERα-positive breast cancer (figure 1.8A), the AR acts as a competitor of ERα and thus suppresses ERα-mediated tumour growth (Birrell *et al.* 1995, Szelei *et al.* 1997, Lanzino *et al.* 2005, Macedo *et al.* 2006, Peters *et al.* 2009, Hickey *et al.* 2012). The above-mentioned is supported by the results from an *in vitro* study by Peters et al. (2009)

showing that in ERα-positive breast cancer cells, the AR inhibits the transactivation function of ERα, as well as ERα-mediated cell proliferation (Peters *et al.* 2009). In ERα-negative breast cancer (figure 1.8B), it is proposed that the AR levels increase and that there are changes in cofactor interactions, resulting in the AR mimicking the function of ERα, thereby sustaining tumour growth (Robinson *et al.* 2011, Hickey *et al.* 2012).

Several mechanisms have been identified whereby the AR inhibits the actions of ERα. For example, it has been shown that the AR can bind directly to EREs (Peters *et al.* 2009, Rizza *et al.* 2014), thereby preventing ERα from interacting with the ERE and as a result inhibiting the transactivation function of ERα (Peters *et al.* 2009, Hickey *et al.* 2012, Lamb *et al.* 2014). Considering that both transactivation and transrepression of ER target genes play a role in the regulation of breast cancer cell survival and proliferation, it would be interesting to know whether the transrepression function of ERα would also be inhibited by the AR. Furthermore, ERα and the AR have been shown to interact with similar co-regulatory molecules (Lanzino *et al.* 2005, Fioretti *et al.* 2014), therefore it is possible that when co-expressed these receptors may compete for these molecules. One such example is the AR associated (ARA70) protein, a known AR co-activator that also acts as an ERα co-activator (Lanzino *et al.* 2005). Lastly, ERα and the AR have similar non-genomic activities. This means that the AR as well as ERα interact with similar accessory proteins such as modulator of non-genomic activity of estrogen receptor (MNAR), which is important for cell membrane actions of these receptors. The ability of the AR to interact with these proteins prevents ERα from associating with these proteins thus leading to ERα being unable to regulate signalling pathways at the cell membrane (Fioretti *et al.* 2014). Unlike, the numerous studies examining the interaction between the AR and ERα in breast cancer, not much is known about the interplay between ERβ and the AR.

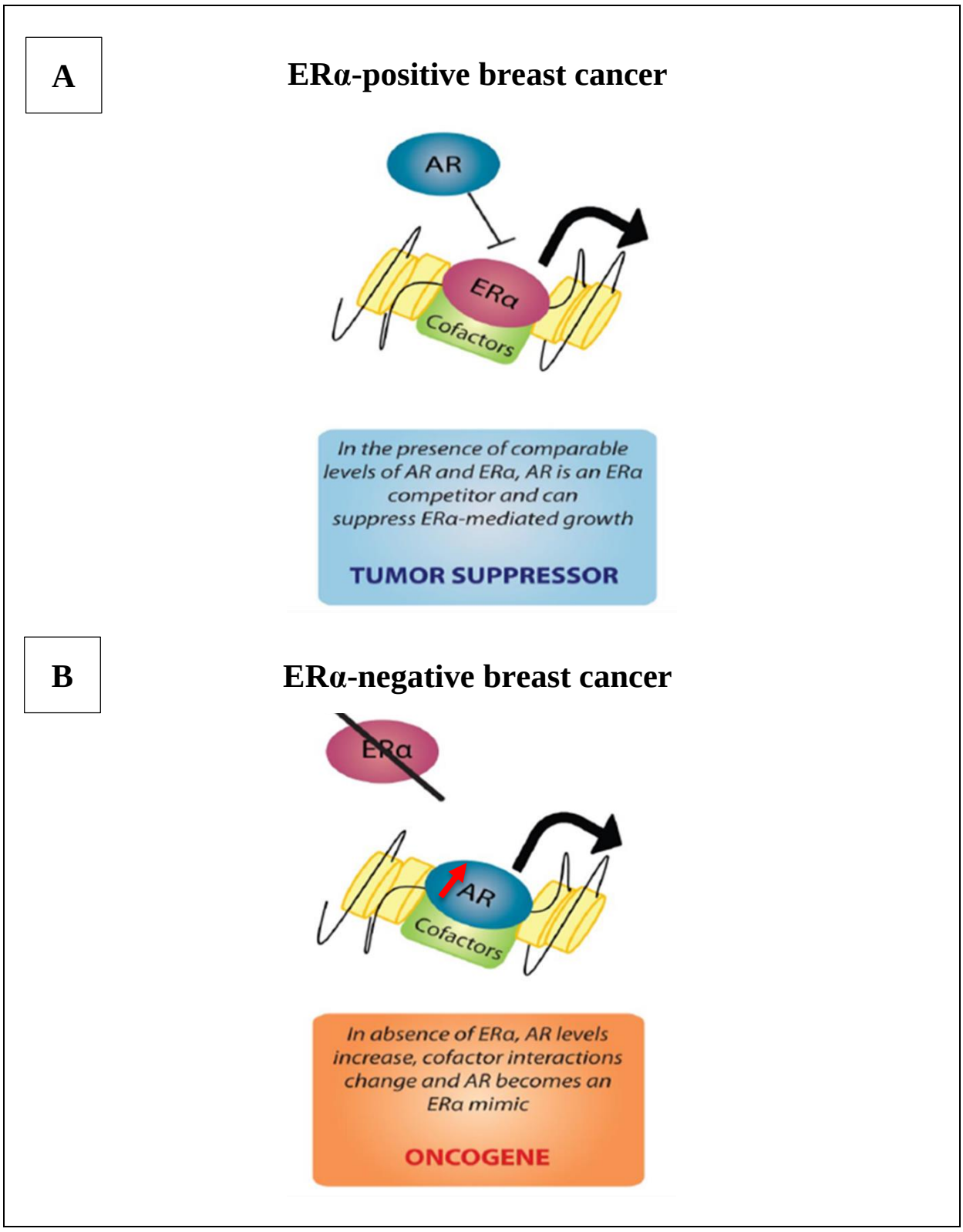

Figure 1.8 Proposed mechanism of the tumour suppressor vs. oncogenic roles of the AR in breast cancer. (A) The AR can act as a tumour suppressor by competing with ERα for binding to EREs, leading to the suppression of ERα-mediated growth. (B) In the absence of ERα, the AR mimics the actions of ERα thereby becoming an oncogene. The red arrow indicates an increase in AR levels. Adapted from (Hickey *et al.* 2012).

1.6. Conclusion

For over a century it has been appreciated that E_2 and the ER play critical roles in breast cancer biology and their role in breast cancer has been extensively studied. ERα has been reported to promote cell growth and survival, while ERβ inhibits ERα-mediated cell proliferation. However, emerging evidence suggests that other steroid receptors, like the AR, are also involved in the regulation of breast cancer growth (Birrell *et al.* 1998, Brys 2000, Hartman *et al.* 2012, Shah *et al.* 2013, Lakis *et al.* 2014). Interestingly, the evidence in the literature suggests that the precise function of the AR in breast cancer is dependent on whether or not ERα is present, with little known about the link between the AR and ERβ. Figure 1.9 summarises the current knowledge on the interplay between the ER subtypes and the AR in breast cancer. The illustration shows that both the AR and ERβ can inhibit cell survival and proliferation, directly and indirectly, by suppressing the activity of ERα (Peters *et al.* 2009, Need *et al.* 2012). Also shown is the fact that not much is known about whether the AR can have an effect on the activity of ERβ. Given that both the AR and ERβ elicits growth-inhibitory effects in the presence of ERα, and that the role of the AR and ERβ changes to promote breast cancer development in the absence of ERα, it is important to elucidate the influence of the AR on the function of ERβ. Furthermore, although it is known that the AR modulates the transactivation function of ERα, one cannot exclude the fact that ERs elicit their biological activity via both transactivation and transrepression mechanisms. Thus, it is important to also study the effects of the AR on the transrepression function of the ER subtypes. This is particularly important in the light of the fact that inflammation is known to promote the development and progression of breast cancer (Niu *et al.* 2012, Bhatelia *et al.* 2014, Suman *et al.* 2015), and repression of NFκB-regulated genes via the ER subtypes potentially reduces the risk of inflammation-induced breast cancer (Visser *et al.* 2013).

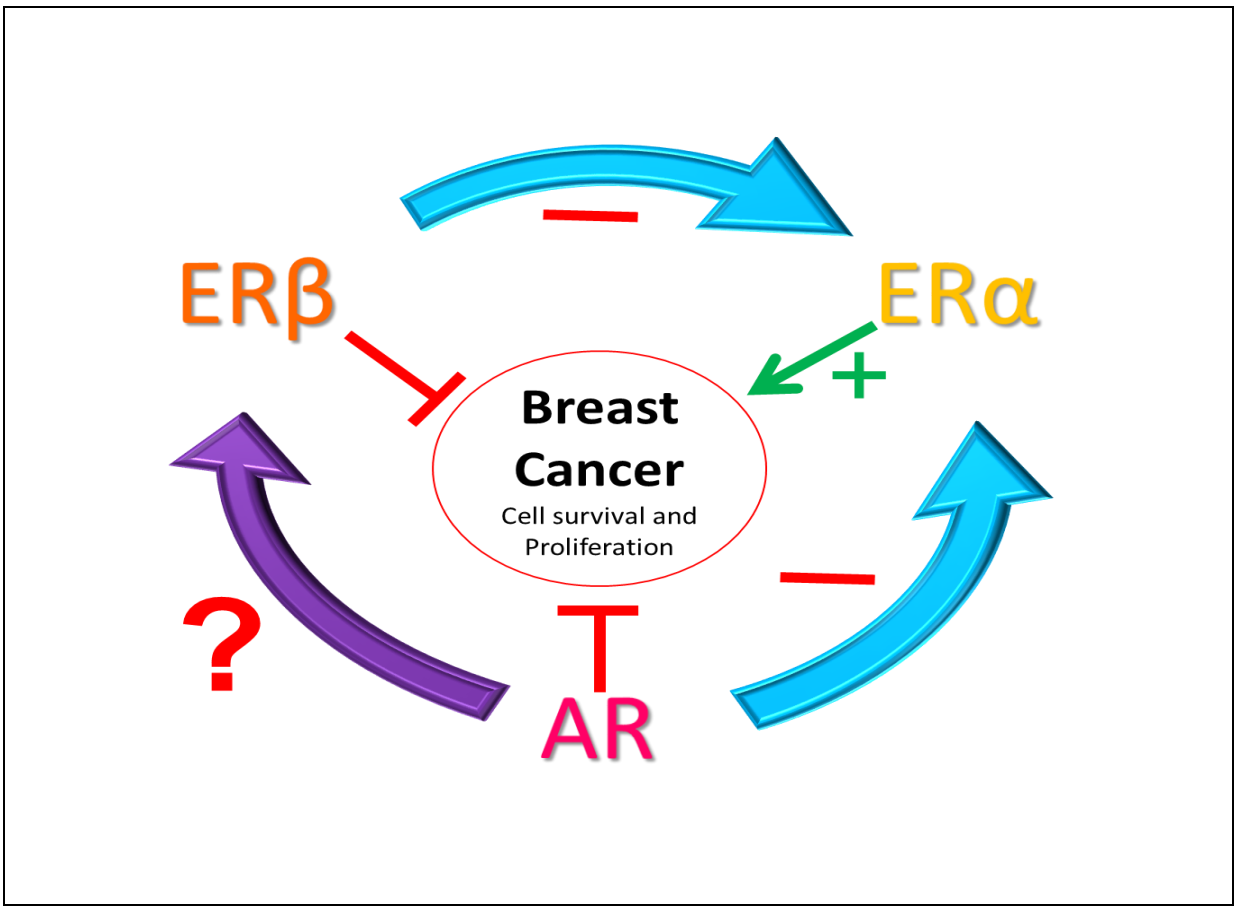

Figure 1.9. Interplay between the ER subtypes and the AR in breast cancer. ERα promotes cell survival and proliferation, while ERβ antagonizes ERα-mediated cell proliferation. The AR has been shown to have the ability to inhibit cell proliferation both directly and indirectly by suppressing the activity of ERα. – and T denote inhibition of activity, + shows activation of activity, the blue arrows demonstrate that the effect is towards ERα while the purple arrow demonstrates that the effect is towards ERβ.

1.7. Aims of the study

The evidence in literature suggests that the AR decreases the transactivation function of ERα and that the decrease is more pronounced in the presence of AR ligand (Peters *et al.* 2009). Furthermore, it has been reported that the AR mimics the function of ERα as it can bind to EREs, but that ERα cannot bind to an ARE. Whether the AR has the same effect on the transactivation function of ERβ and whether the latter can transactivate via an ARE, is not well studied. Moreover, considering that ERs have both transactivation and transrepression

functions, studies investigating the influence of the AR on the transrepression function of both ERα and ERβ are lacking.

Thus, the main aim of the current study was to investigate the influence of the AR on both the transactivation and transrepression functions of the ER subtypes, with the main focus being on ERβ. The ER- and AR-deficient MDA-MB-231 human breast cancer cell line was used as an *in vitro* model system for breast cancer to investigate the following:

1. The validation of the experimental system used by Peters *et al*. (2009).

2. The effect of increasing concentrations of human AR, in the absence and presence of AR ligands, on the transactivation function of human ERβ via a synthetic ERE-containing promoter reporter construct.

3. The effect of increasing concentrations of the AR on the transactivation function of both ER subtypes on an endogenous ER-regulated gene.

4. The ability of both ER subtypes to transactivate via a synthetic and an endogenous ARE-containing promoter.

5. The effect of the AR on the transrepression function of both ER subtypes via a synthetic NFκB-containing promoter.

6. The ability of the AR to modulate ERα- or ERβ-mediated cell proliferation.

Chapter 2
Materials and Methods

2.1. Plasmids

The plasmids expressing the human ERα (pSG5-ERα) and ERβ (pSG5-ERβ) were received from Prof. Frank Gannon (European Molecular Biology Laboratory, Heidelberg, Germany) (Gilles *et al.* 2000). A plasmid expressing the human AR, $pSVAR_o$, (Kastner *et al.* 1990) was obtained from Prof Frank Claessens (University of Leuven, Belgium) (Brinkmann *et al.* 1989). The promoter-reporter construct vit-ERE-TATA-luciferase containing two copies of the estrogen response element (ERE) previously described by (Hall *et al.* 2002) was obtained from Prof Ken Korach (National Institute of Environmental Health Sciences, North Carolina, USA), while the $p(IL6\kappa B)_3$50hu.IL6Pluc$^+$ promoter reporter construct was obtained from Prof. Guy Haegeman (University of Gent, Belgium) (Plaisance *et al.* 1997). The pGL2 basic vector, purchased from Promega (Madison, USA), was used as a filler plasmid and the pTAT-GRE-E1b-luc, driven by the E1b promoter containing two copies of the rat TAT-GRE, was obtained from Dr. G. Jenster (Erasmus University of Rotterdam, Netherlands) (Sui *et al.* 1999). The latter promoter-reporter construct will be referred to as an ARE-luc.

2.1.1. Plasmid DNA preparation

Escherichia coli DH5α cells were made competent using the calcium chloride method and transformed using the heat shock method (Sambrook *et al.* 1989). A starter culture was prepared by growing a single colony of the transformed bacteria in 5 ml Luria-Bertani (LB) medium in the presence of 50 µg/ml ampicillin (all plasmid vectors used in this study (mentioned in 2.1) contain an ampicillin-resistant gene) for 6 hours while shaking at 37°C. Thereafter 5 ml of the starter cultures were transferred into 250 ml fresh LB and 50 µg/ml ampicillin. The latter was incubated for 16 hours at 37°C to allow cells to reach exponential growth. Plasmid DNA was purified using the PureYield^{TM} Plasmid Maxiprep System (Promega, Madison, USA) according to the manufacturer's instructions. Briefly, the cultures were centrifuged for 10 minutes at 5000

x g and the supernatants discarded. Each pellet was resuspended in 12 ml Cell Resuspension Solution, before lysis in 12 ml Cell Lysis Solution. Lysis was stopped by adding 12 ml Neutralization Solution, followed by centrifugation of the lysate at 14 000 x g for 30 minutes. Two columns were used to purify the plasmid DNA using a vacuum manifold. The PureYield™ clearing column was set on top of the PureYield™ maxi binding column. The lysate was decanted into the PureYield™ clearing column and maximum vacuum was applied. The PureYield™ clearing column was removed after the lysate had passed through to the PureYield™ maxi binding column. The latter was then washed with 5 ml Endotoxin Removing Buffer, followed by 20 ml Column wash while applying maximum vacuum. The column was subsequently dried for 5 minutes under vacuum, and then inserted into a 50 ml tube. Nuclease-free water was added to the column prior to centrifugation in a swing bucket rotor at 2000 x g for 5 minutes. The eluate containing the plasmid DNA was collected and the DNA concentration determined using a Nanodrop 1000 (Thermo Fisher Scientific, South Africa). Restriction enzyme digests and agarose gel electrophoresis were used to determine the size and integrity (quality) of the plasmid DNA.

2.2. Test compounds

The test compounds used in this study included 17β-estra-1,3,5(10)-etriene-3,17 diol [17-β-estradiol; (E_2)], 5α-androstan-17β-ol-3-one [5α–dihydrotestosterone; (DHT)], 6α-methyl-17α-hydroxyprogesterone acetate [medroxyprogesterone acetate; (MPA)] and 17α-ethynyl-19-nortesterone 17β-acetate [norethisterone acetate; (NET-A)]. All the test compounds and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma-Aldrich, South Africa. Test compounds were prepared in absolute ethanol (EtOH) and stored in glass vials at -20^{o}C.

2.3. Cell culture

The human breast cancer cell line, MDA-MB-231 (a kind gift from Prof. Guy Haegemann, University of Gent, Belgium), was maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, South Africa) containing 4.5 g/ml glucose, phenol-red and supplemented with 10% (v/v) fetal calf serum (FCS) (Separations, South Africa), 100 IU/ml penicillin (Sigma-Aldrich, South Africa) and 100 µg/ml streptomycin (Sigma-Aldrich, South Africa). COS-1 cells (African monkey kidney fibroblast cells), obtained from the American Type Culture Collection (ATCC, Virginia, USA), were maintained in DMEM containing phenol red supplemented with 10% (v/v) FCS, penicillin (100 IU/ ml) and streptomycin (100 µl/ml). All the cell lines were maintained in 75 cm^2 flasks (Greiner Bio-One International, Austria) at 37^{o}C with a relative humidity of 90% and 5% CO_2. The cell lines were regularly tested for mycoplasma infection using Hoechst staining (Freshney 1987), and only mycoplasma negative cells were used in experiments.

2.4. Promoter reporter assays

2.4.1. Transactivation

MDA-MB-231 cells were seeded into 96-well plates (Porvair Science, United Kingdom) at a density of 1.75 x 10^4 cells per well in DMEM containing phenol red, 10% (v/v) FCS, 100 IU/ml penicillin and 100 µg/ml streptomycin. The cells were then incubated for 24 hours to allow complete adherence to the surface of the plates. The following day, the cells were rinsed once with sterile 1x PBS and the medium was replaced with phenol red-free DMEM supplemented with 5% (v/v) charcoal treated FCS, 100 IU/ml penicillin and 100 µg/ml streptomycin. Phenol-red free DMEM was used because it is well-known that phenol-red has estrogenic activity (Welshons *et al.* 1988). The cells were subsequently transiently transfected with either 100 ng of the vit-ERE-TATA-luciferase construct and 2.5 ng of the expression vector for human ERα (pSG5-hERα) or 50 ng vit-ERE-TATA-luciferase construct and 2.5 ng of the expression vector

for human ERβ (pSG5-hERβ), in the absence or presence of 2.5, 5 or 10 ng of the expression vector for human AR (pSVAR$_o$), using X-tremeGENE HP DNA transfection reagent (Roche Molecular Biochemicals, South Africa) according to the manufacturer's instructions. The filler plasmid pGL2 basic was used to ensure a constant total DNA concentration of 112.5 ng. Transfection and subsequent treatment conditions were similar to that described in Peters *et al*. (2009). Briefly, 3 hours after transfection, the cells were incubated with 0.1% (v/v) EtOH (control) or 1 nM E_2, DHT, MPA or NET-A (agonist mode), or 1 nM E_2 in the absence or presence of 1 nM DHT, MPA or NET-A (antagonist mode) for 36 hours. The cells were subsequently washed with sterile 1x PBS and lysed by adding 25 µl passive lysis buffer (0.2% (v/v) Triton-X 100, 10% (v/v) glycerol, 2.8% (v/v) Tris-phosphate-ethylenediaminetetra-acetic acid (EDTA) and 1.44 mM EDTA) per well. The plates were then placed on a shaker for 15 minutes at room temperature, before being frozen at -20^{o}C, as the freeze-thawing facilitates effective lysis. On thawing of samples, 10 µl of the lysates were assayed for luciferase activity using the Luciferase Assay system (Promega luciferase assay reagent, Madison, USA) and a Veritas microplate luminometer (Turner biosystems, USA). In addition, 5 µl of the cell lysate was used to determine the protein concentration by the Bradford protein determination (Bradford 1976). The luciferase values obtained for all experiments (expressed as relative light units (RLUs)) were normalised relative to the protein concentration for each sample. Some results are expressed as fold induction with the EtOH value set as one, while other results are expressed as the fold induction value in the presence of 1 nM E_2 set as 100% with all other values calculated relative to that. The reported values are averages of at least 3 independent experiments, with each condition performed in triplicate.

2.4.2. Transrepression

The MDA-MB-231 cell line was seeded into 96-well plates at a density of 1.75 x 10^4 cells per well in DMEM containing phenol-red. After 24 hours, the cells were transiently transfected with 2.5 ng of either pSG5-hERα or pSG5-hERβ and 100 ng p(IL6κB)$_3$50hu.IL6Pluc$^+$, in the absence or presence of 2.5, 5 or 10 ng pSVAR$_o$, using the X-tremeGENE HP DNA transfection reagent (Roche Molecular Biochemicals, South Africa) according to the manufacturer's instructions. The filler plasmid, pGL2 basic, was used to ensure a constant total DNA concentration of 112.5 ng. After 3 hours the cells were incubated with 0.1 % (v/v) EtOH in the absence or presence of 10 ng/ml PMA. For agonist mode, cells were treated with 10 ng/ml PMA in the absence (0.1% (v/v) EtOH) or presence of 1 nM E_2, DHT, MPA or NET-A for 36 hours. For antagonist mode, cells were treated with 1 nM E_2 in the absence and presence of 1 nM DHT, MPA or NET-A for 36 hours. Cells were lysed and analysed as in 2.4.1. The luciferase values obtained for all experiments were normalized relative to the protein concentration for each sample. Results are expressed as either the fold induction with the EtOH value set as one in order to quantify the extent of PMA induction, or as normalised percentage response with the value for EtOH + PMA set as 100% in order to determine the extent of repression obtained.

2.5. Western blot analysis

To determine the steroid receptor content in the MDA-MB-231 human breast cancer cell line, cells were plated into 6-well plates (Porvair Science, United Kingdom) at a density of 2.5 x 10^5 cells per well. For positive controls, COS-1 cells were seeded into 12-well plates (Porvair Science, United Kingdom) at a density of 1 x 10^5 cells per well and transiently transfected with 250 ng of the respective steroid receptor expression vector for human ERα, ERβ or AR (pSG5-ERα, pSG5-ERβ or pSVAR$_o$). Untransfected COS-1 cells were used as negative controls. After 48 hours, the cells were washed with 1x PBS and placed on ice, before lysis with 80 µl (6 well

plate) or 100 µl (12 well plate) 2x sodium dodecyl sulphate (SDS) sample buffer (1 M Tris-hydrogen chloride (HCl) (pH 6.8), 10% (w/v) SDS, 0.1% (w/v) bromophenol blue, 2% (v/v) β-mercaptoethanol and 20% (v/v) glycerol) (Sambrook *et al.* 1989). Cells were removed from the wells using a cell scraper and then transferred into a 1.5 ml Eppendorf tube (Eppendorf AG, Hamburg, Germany). All lysates were denatured by boiling for 10 minutes at 97^{o}C. Protein samples were separated on a 10% SDS-polyacrylamide gel by performing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) at 100 V for 15 minutes and 200 V for 45 minutes using 1x SDS running buffer (3.5 mM SDS, 25 mM Tris-HCl, 192 mM Glycine). After electrophoresis, the samples on the acrylamide gel were transferred onto a hybond Enhanced Chemiluminescence (ECL) nitrocellulose membrane (Amersham Biosciences, USA) at 180 mA for 90 minutes using 1x transfer buffer (25 mM Tris-HCl (pH 7), 192 mM glycine and 10% methanol). The membranes were blocked for 2 hours in 10% (w/v) fat-free milk powder in 1x TBS (Tris Buffered Saline - 50 mM Tris-HCl and 150 mM NaCl) containing 0.1% (v/v) Tween-20 (TBST). Thereafter, the membranes were rinsed with 1x TBST and incubated with specific primary antibodies directed against the steroid receptors of interest (Santa Cruz Biotechnology Inc, Europe), for 16 hours at 4^{o}C. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. The membranes were then washed with TBST (once for 15 minutes and three 5 minute washes), before incubating with horseradish peroxidase (HRP)-conjugated secondary antibodies (goat anti-rabbit or anti-mouse) (Santa Cruz Biotechnology Inc., Europe) at room temperature for 90 minutes. The dilutions for all antibodies used in this study are summarised in Table 2.1. The membranes were washed again as indicated above, and then visualized using Clarity^{TM} ECL western blotting substrate (Biorad, South Africa) and medical X-ray film (Africa X-Ray Industrial and Medical (Pty) LTD., South Africa).

Table 2.1 Primary and secondary antibody dilutions used for Western blotting

Primary antibody	Dilution	HRP-conjugated secondary antibody	Dilution
ERα (M-20)	1:500	goat anti-rabbit	1:1 000
ERβ (H-150)	1:1 000	goat anti-rabbit	1:1 000
AR (441)	1:2 000	goat anti-mouse	1:5 000
GR (H-300)	1:4 000	goat anti-rabbit	1:5 000
MR (H-300)	1:500	goat anti-rabbit	1:5 000
PR-A/B (AB-52)	1:500	goat anti-rabbit	1:2 000
GAPDH (0411)	1:4 000	goat anti-mouse	1:15 000

2.6. Total RNA isolation and complementary DNA (cDNA) synthesis

Human MDA-MB-231 cells were seeded into 12-well plates at a density of 1 x 10^5 cells per well. Cells were transfected with 33.3 ng of either pSG5-hERα or pSG5-hERβ expression vectors in the absence and presence of increasing concentrations of the $pSVAR_o$ (0, 33.3, 66.6 and 133.2 ng, respectively) and pGL2 basic expression vectors to ensure a constant total DNA concentration of 166.5 ng, using X-tremeGENE HP DNA transfection reagent (Roche Molecular Biochemicals, South Africa) according to the manufacturer's instructions. Twenty four hours later the cells were treated for 12 hours with either 0.1% (v/v) EtOH, 1 nM E_2 or DHT, or equimolar amounts of both E_2 and DHT in phenol red-free DMEM supplemented with 5% (v/v) charcoal treated FCS. Following a single PBS wash, the total RNA was isolated from the cells using Tri-reagent (Sigma-Aldrich, South Africa) according to the manufacturer's instructions. Briefly, the cells were lysed by adding 400 µl Tri-reagent and stored overnight at -80°C, as the freeze-thawing facilitates lysing of the cells. On thawing, the lysates were

transferred to 1.5 ml microcentrifuge tubes and incubated for 5 minutes at room temperature. Thereafter 80 µl chloroform was added and the samples were vortexed for 1 minute. The samples were incubated for 3 minutes at room temperature before centrifugation at 20 800 x g for 15 minutes at 4°C. The aqueous phase was transferred to a clean microcentrifuge tube and an equal volume of ice-cold isopropanol was added. Samples were vortexed for 1 minute and incubated for 15 minutes at room temperature, followed by centrifugation at 20 800 x g for 15 minutes at 4°C to pellet the RNA. The pellets were subsequently washed by adding 500 µl 75% (v/v) EtOH, vortexing for 1 minute, and centrifugation at 6800 x g for 5 minutes at 4°C. The supernatant was aspirated and the RNA pellets were air-dried for 10 minutes on ice before dissolving pellets in 15 µl diethyl pyrocarbonate (DEPC)-treated water. The RNA concentration was measured using a Nanodrop ND-100 Spectrophotometer (Inqaba Biotec, South Africa) and the $A_{260/280}$ ratios were determined. All RNA samples were pure as indicated by $A_{260/280}$ ratios between 1.9 and 2.1 in a 10 mM Tris buffer (Sambrook *et al.* 1989). The integrity of the RNA was confirmed on 1% (w/v) denaturing formaldehyde agarose gels by the presence of intact 28S and 18S ribosomal bands. All RNA samples were stored at -80°C. The total RNA extracted from the transfected MDA-MB-231 cell line was reverse transcribed using the transcriptor first strand cDNA synthesis kit (Roche Applied Science, South Africa) according to the manufacturer's instructions. Briefly, 0.5 µg total RNA was added to 2.5 µM anchored-oligo$(dT)_{18}$-primer and if needed, PCR-grade water was added to a final volume of 6.5 µl. The template-primer mixture was incubated at 65°C for 10 minutes to denature the RNA secondary structures and immediately placed on ice. Thereafter the following was added: 2 µl of 1x transcriptor reverse transcriptase reaction buffer (8 mM $MgCl_2$), 0.25 µl of protector RNase inhibitor (20 U/µl), 1 µl of deoxynucleotide mix (1 mM per nucleotide) and 0.25 µl of transcriptor reverse transcriptase (10 U/µl) (Roche Applied Science, South Africa). The samples were then incubated at 50°C for an hour in order for reverse transcription to take place.

The reaction was stopped by incubating the samples at 85ºC for 5 minutes in order to inactivate the transcriptor reverse transcriptase. The cDNA samples were stored at –20ºC.

2.7. Realtime quantitative polymerase chain reaction (qPCR)

Realtime qPCR was performed using the StepOne Plus Real Time PCR system (Applied Biosystems, South Africa) according to the manufacturer's instructions. The qPCR reaction mixture was prepared by adding a final concentration of 0.2 µM forward and reverse primers respectively (table 2.2), 5 µl 1x SYBR Green (Kapa biosystems, South Africa) and PCR-grade water to a final volume of 9 µl per sample. Thereafter, 1 µl cDNA template or PCR-grade water (negative control) was added. The samples were incubated for 3 minutes at 95ºC to activate the Taq polymerase, followed by denaturation for 15 seconds at 95ºC. The thermal cycling profiles for PR, PSA (prostate specific antigen) and GAPDH genes are summarized in table 2.3. Agarose gel electrophoresis and melting curve analysis were used to confirm the presence of a single amplicon. A cDNA dilution series was used to generate a standard curve to determine the amplification efficiency of each primer set (Addendum). The efficiencies were 1.96, 1.94 and 2.12 for PR, PSA and GAPDH, respectively. The relative transcript levels of target genes were normalized to the relative transcript levels of GAPDH by using the mathematical model described by (Pfaffl 2001).

Table 2.2. Primer sequences for genes investigated in this study.

Gene	Primers (5′ – 3′)	Strand	Amplicon size (bp)	Reference
PR	CTTAATCAACTAGGCGAGAG AAGCTCATCCAAGAATACTG	Forward Reverse	122	(Kocanova *et al.* 2010)
PSA	AGGCCTTCCCTGTACACCAA GTCTTGGCCTGGTCATTTCC	Forward Reverse	133	(Bohrer *et al.* 2010)
GAPDH	TGAACGGGAAGCTCACTGG TCCACCACCCTGTTGCTGTA	Forward Reverse	307	(Ishibashi *et al.* 2003)

Table 2.3. Thermal cycling profiles for all genes investigated.

Gene	Annealing		Elongation		Number of thermal cycles
	Temperature (°C)	Time (seconds)	Temperature (°C)	Time (seconds)	
PR	58	30	72	20	40
PSA	60	10	72	10	45
GAPDH	59	10	72	12	28

2.8. Methyl Thiazolyl Tetrazolium (MTT) cell proliferation assay

The MDA-MB-231 cell line was seeded into 96-well plates at a density of 1 x 10^4 cells per well. The following day, cells were washed with 200 µl pre-warmed PBS (37^{o}C) followed by the addition of 200 µl phenol-red free DMEM, supplemented with 10% (v/w) charcoal-stripped FCS. The cells were subsequently transfected with either 2.5 ng pSG5-hERα, or pSG5-hERβ or pGL2 basic using X-tremeGENE HP DNA transfection reagent according to the manufacturer's instructions. After 24 hours, the medium was aspirated and the cells were treated for 48 hours with 0.1% (v/v) (EtOH) or 1 nM E_2 in phenol-red free DMEM supplemented with 10% (v/w) charcoal-stripped FCS only. After 48 hours, 50 µl of the colorimetric MTT solution (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich, South Africa) prepared by dissolving 5 mg/ml MTT in pre-warmed PBS and filter-sterilising the solution, was added to each well together with 150 µl phenol red free DMEM containing 10% (v/w) charcoal-stripped FCS. The yellow MTT tetrazolium salt is reduced by metabolically active cells to an insoluble purple formazan precipitate that can be solubilized with dimethyl sulfoxide (DMSO) and quantified spectrophotometrically. After a 4 hour incubation with the MTT solution, the medium was aspirated and 200 µl DMSO was added to each well to solubilize the crystals. The plate was covered with foil and incubated at room temperature for 5 minutes, while shaking. The absorbance was then read at 540 nm in a Power wave 340 microtiter plate reader (Biotek, Winooski, USA). Results were expressed as fold induction with the value for EtOH set as one.

2.9. Data manipulation and statistical analysis

The GraphPad Prism® Software version 5.3 was used for data manipulation, graphical representations and statistical analysis. Two-way ANOVA analysis of variance and Bonferroni's (compares all pairs of columns) post-tests was used for statistical analysis of

grouped data. Unpaired t-tests were used for column graphs with only two sets of data. One-way ANOVA analysis of variance with Bonferroni's (compare all pairs of columns) post-tests as well as Newman-Keuls (compare all pairs of columns) post-tests was used for all column graphs with more than two sets of data. Statistically significant differences are indicated by (*, **or ***) to indicate $p<0.05$, $p<0.01$ or $p<0.001$, respectively, whereas no statistical difference, ($p>0.05$) is indicated by ns. The letters a, b and c are used to indicate statistical significant differences, with values differing from each other being assigned different letters.

Chapter 3
Results

3.1. Validating the experimental system

A study by Peters *et al*. (2009) showed that the unliganded AR decreases the transcriptional activation of ERα, and that in the presence of liganded AR, this decrease was in a concentration dependent manner. This raised the question of whether the AR could decrease the activity of ERβ in a similar manner. To address this question, the model system used by Peters and co-workers was firstly validated, by following similar transfection and treatment conditions in the same cell line (see chapter 2, section 2.4.1). Briefly, the ER and AR negative human MDA-MB-231 breast cancer cell line (absence confirmed by western blot in figure 3.1) was transiently transfected with an ERα expression vector and an estrogen response element (ERE)-driven reporter construct containing two copies of the ERE, in the absence or presence of increasing concentrations of an AR expression vector.

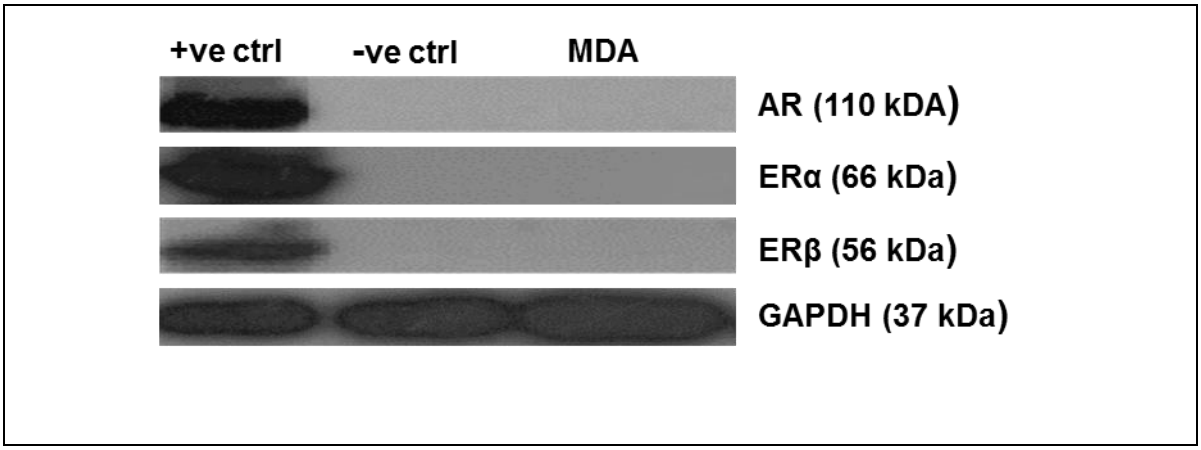

Figure 3.1. The ER subtypes and the AR are not expressed in the MDA-MB-231 breast cancer cell line. Whole cell extracts were prepared from the human MDA-MB-231 (MDA) cell line. Untransfected COS-1 cells were used as the negative control (–ve ctrl), COS-1 cells transiently transfected with pSG5-hERα, pSG5-hERβ or pSVARo expression vectors, respectively were used for the positive controls (+ve ctrl). Protein lysates were analysed by western blotting using antibodies specific to ERα, ERβ, AR or GAPDH, respectively. GAPDH was used as the loading control.

The cells were subsequently treated with 1 nM E_2, the most biologically active estrogen (Samavat and Kurzer 2015). As expected and shown in figure 3.2A, 1 nM E_2 caused significant

transactivation ($p<0.001$) of an ERE-containing promoter-reporter construct via ERα (3.8-fold). Consistent with the results shown by Peters and co-workers, the result in figure 3.2B shows that the AR inhibits E_2-induced ERα transactivation. However, it should be noted that the current study shows a concentration-dependent decrease in the presence of the unliganded AR, and that the decrease is more pronounced than the decrease observed by Peters *et al.* (2009). Furthermore, to exclude a mechanism whereby the AR decreases ERα protein expression, we, like Peters and co-workers, used western blot analysis to show that increasing concentrations of the AR do not affect ERα protein levels (figure 3.3).

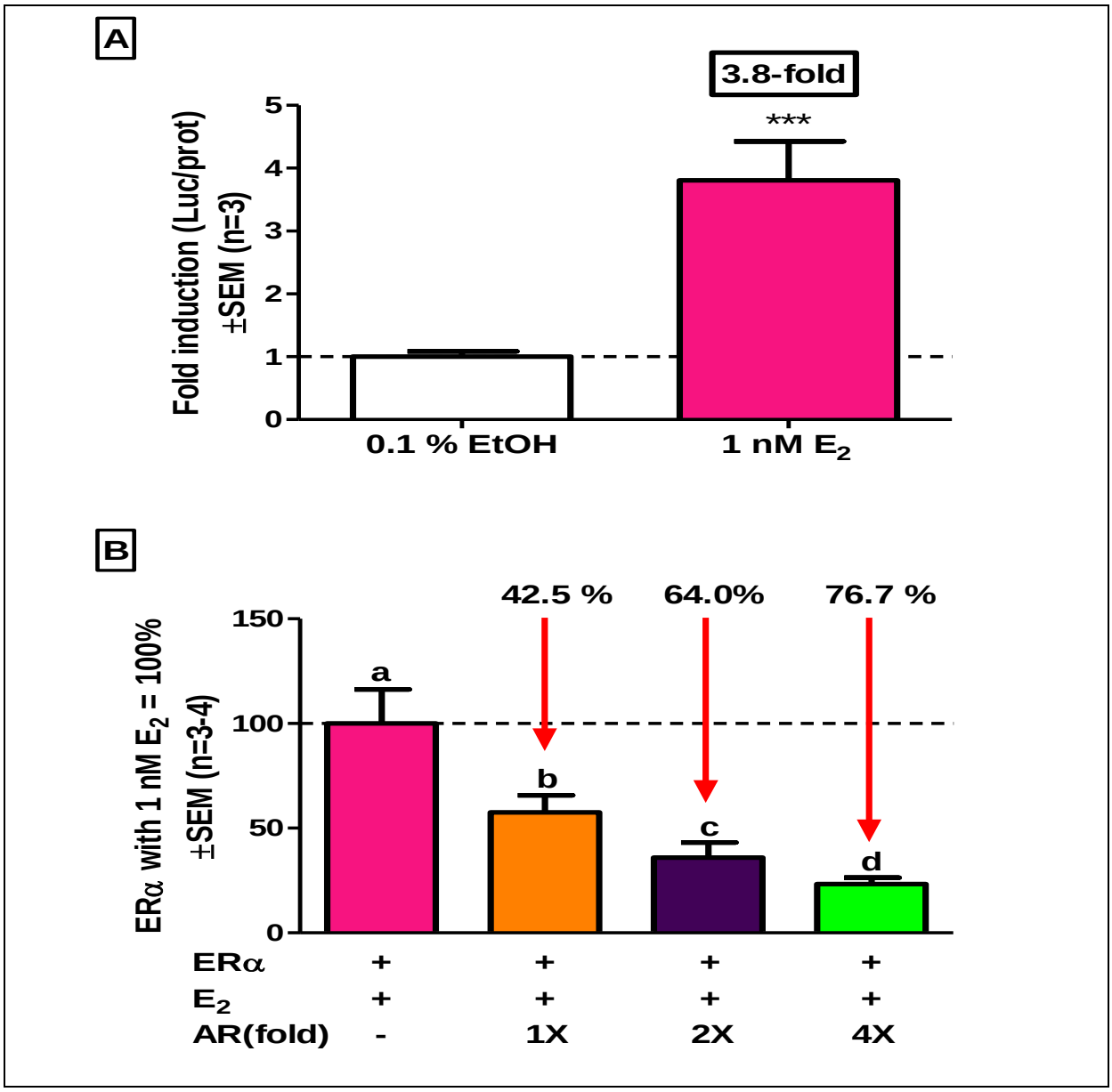

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Figure 3.2. The AR decreases E_2-induced ERα transactivation function. Human MDA-MB-231 breast cancer cells were transiently transfected with pSG5-hERα and the vit-ERE-TATA-luciferase reporter construct, in the absence or presence of increasing concentrations of $pSVAR_O$ (1x, 2x or 4x molar excess), and where needed pGL2basic, using X-tremeGENE HP DNA transfection reagent according to the manufacturer's instructions. After 3 hours cells were treated with 0.1% (v/v) EtOH (control) or 1 nM E_2 for 36 hours. The luciferase values were normalised to the protein concentrations, and expressed as luciferase/protein (luc/prot). The fold induction at 1 nM E_2 via ERα is shown in **(A)**, while in **(B)** the fold induction in (A) was set as 100%, with induction in the presence of various concentrations of AR calculated relative to that. The results indicate the average (± SEM) of at least three independent experiments, with each condition performed in triplicate. Statistical analysis was performed using the unpaired t-test (A) and one-way ANOVA analysis of variance with Bonferroni (compare all pairs of columns) (B) post-tests.

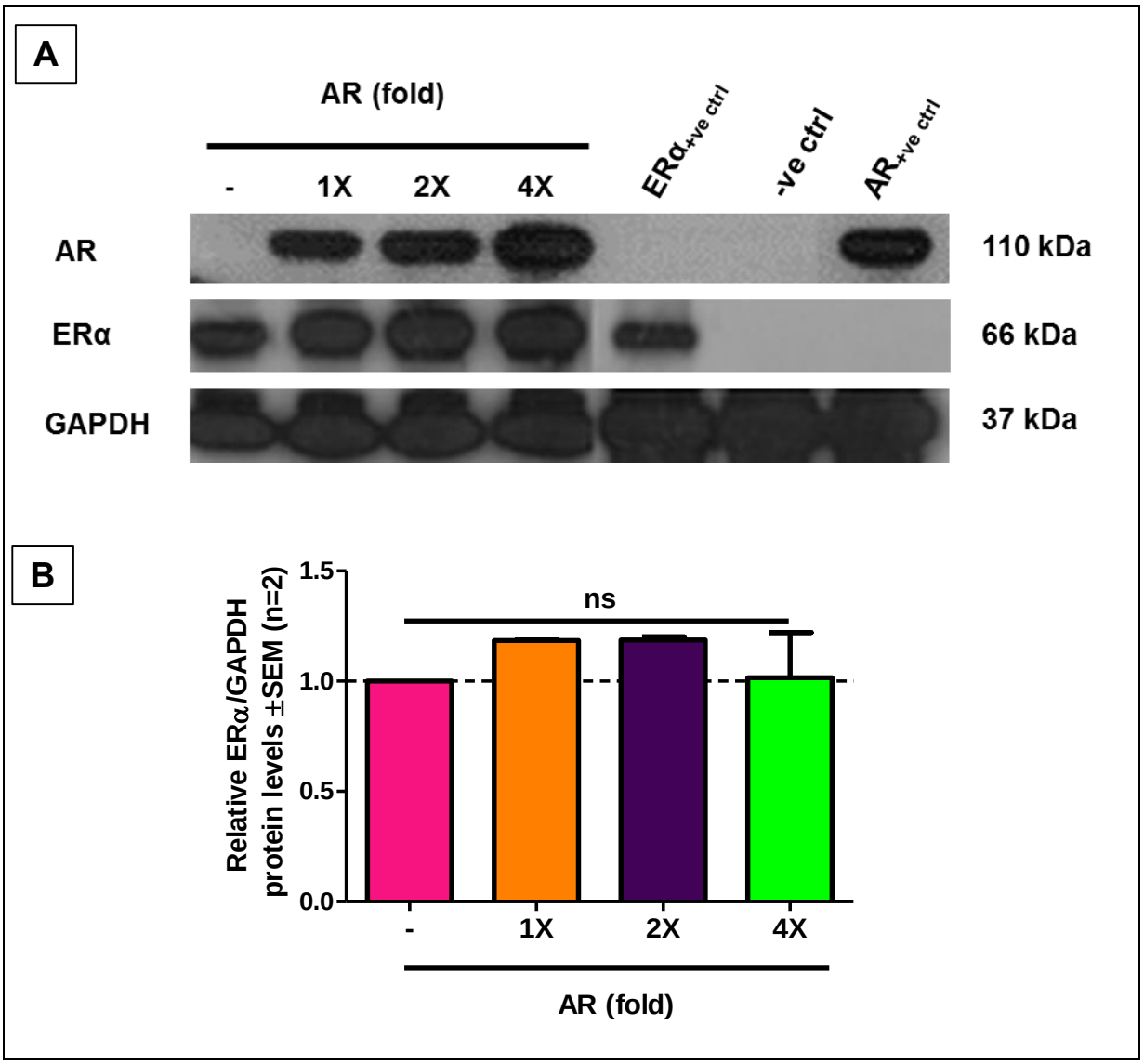

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Figure 3.3. The overexpressed AR does not affect the protein levels of overexpressed ERα in the MDA-MB-231 breast cancer cell line. (A) Human MDA-MB-231 breast cancer cells were transiently transfected as in figure 3.2. Protein lysates were analysed by western blotting with antibodies specific to ERα, AR or GAPDH (loading control). Protein lysates from COS-1 cells transfected with pSG5-hERα and $pSVAR_0$ were used as positive controls (+ve), while lysates from untransfected COS-1 cells were used for the negative control (–ve ctrl). **(B)** Western blots were quantified using UN-SCAN-IT software.

3.2. The AR does not affect the transcriptional activation of ERβ on a synthetic ERE-containing promoter

As the experimental system had been validated, we next investigated the effect of increasing concentrations of the AR on ERβ protein levels as well as on ERβ transcriptional activation. The human MDA-MB-231 cell line was transiently transfected with 2.5 ng pSG5-ERβ and 50 ng vit-ERE-TATA-luciferase in the absence or presence of increasing concentrations of pSVARo (2.5 ng, 5 ng or 10 ng) and where needed, pGL2basic, to ensure that the total amount of DNA transfected remained constant. The cells were subsequently treated with 1 nM E_2 for 36 hours. The western blot in figure 3.4 indicates that, similar to the observation for ERα (figure 3.3), the AR does not affect ERβ protein levels. Having shown that the AR does not affect ERβ protein levels, the effects of increasing concentrations of the AR on the transcriptional activation of ERβ was next investigated. The result in figure 3.5A shows that 1 nM E_2 caused significant transactivation ($p<0.001$) of an ERE-containing promoter-reporter construct via ERβ. However, in contrast to the results obtained for ERα (figure 3.2B), the AR does not affect E_2-induced transcriptional activation via ERβ (figure 3.5B).

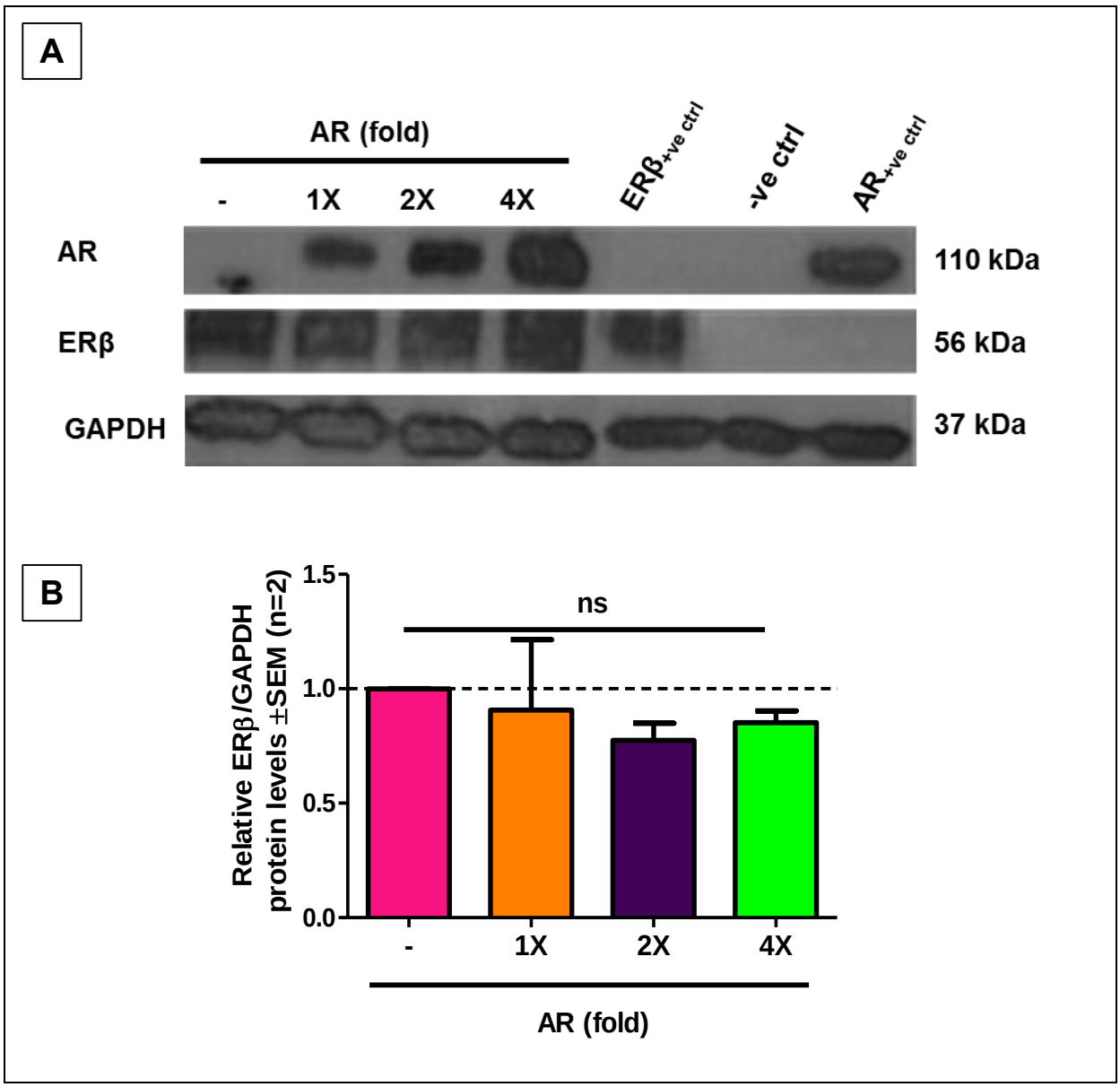


Figure 3.4. The overexpressed AR does not affect protein levels of overexpressed ERβ in the MDA-MB-231 human breast cancer cell line. (A) Human MDA-MB-231 breast cancer cells were transiently transfected with pSG5-hERβ and the vit-ERE-TATA-luciferase reporter construct, in the absence or presence of increasing concentrations of $pSVAR_{O,}$ (1x, 2x or 4x molar excess), and where needed pGL2basic, using X-tremeGENE HP DNA transfection reagent according to the manufacturer's instructions. After 3 hours cells were treated with 0.1% (v/v) EtOH (control) or 1 nM E_2 for 36 hours. Protein lysates were analysed by western blotting with antibodies specific to ERβ, AR or GAPDH (loading control). Protein lysates from COS-1 cells transfected with pSG5-hERβ and $pSVAR_0$ were used as positive controls (+ve), while lysates from untransfected COS-1 cells were used for the negative control (–ve ctrl). **(B)** Western blots were quantified using UN-SCAN-IT software.

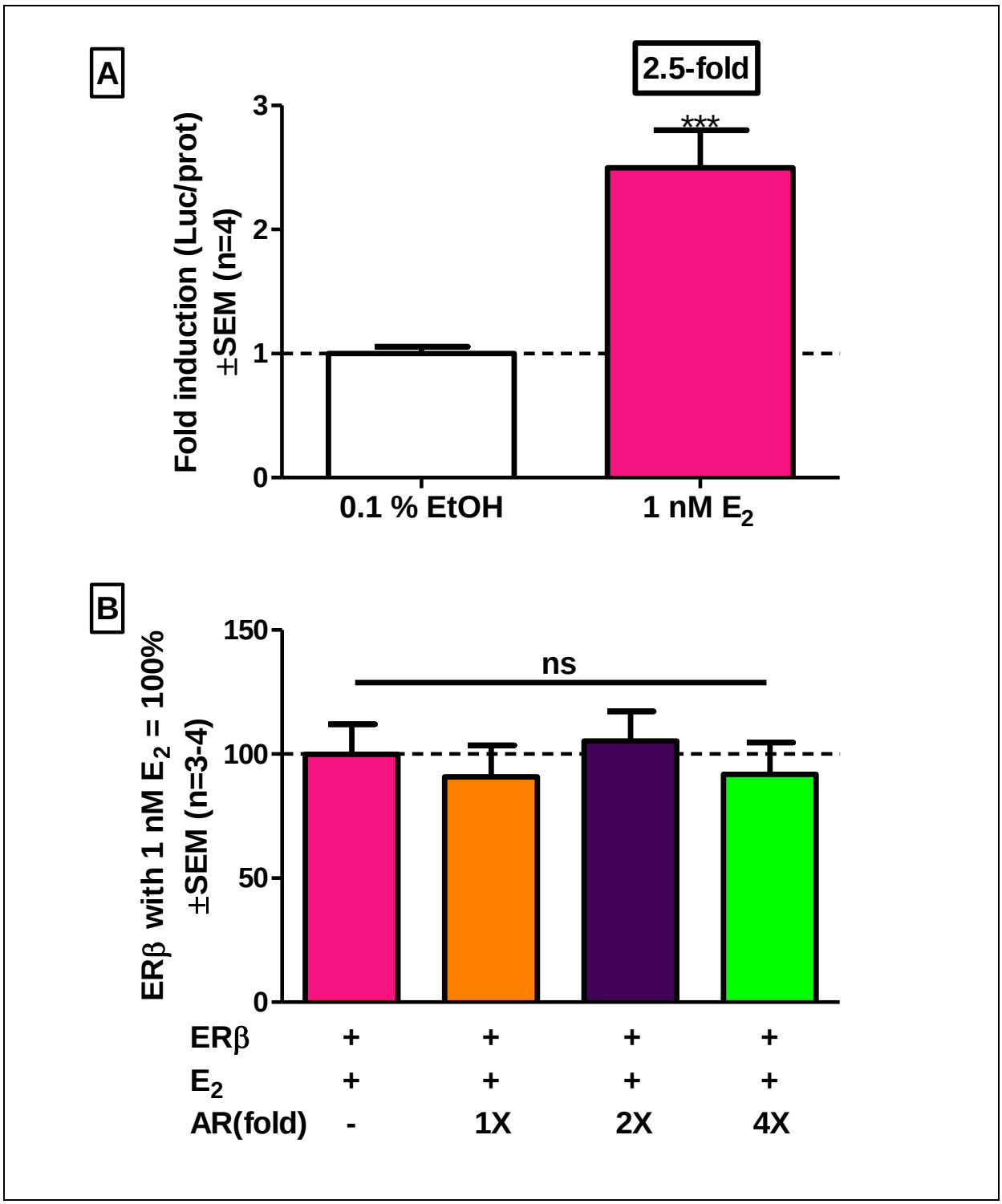
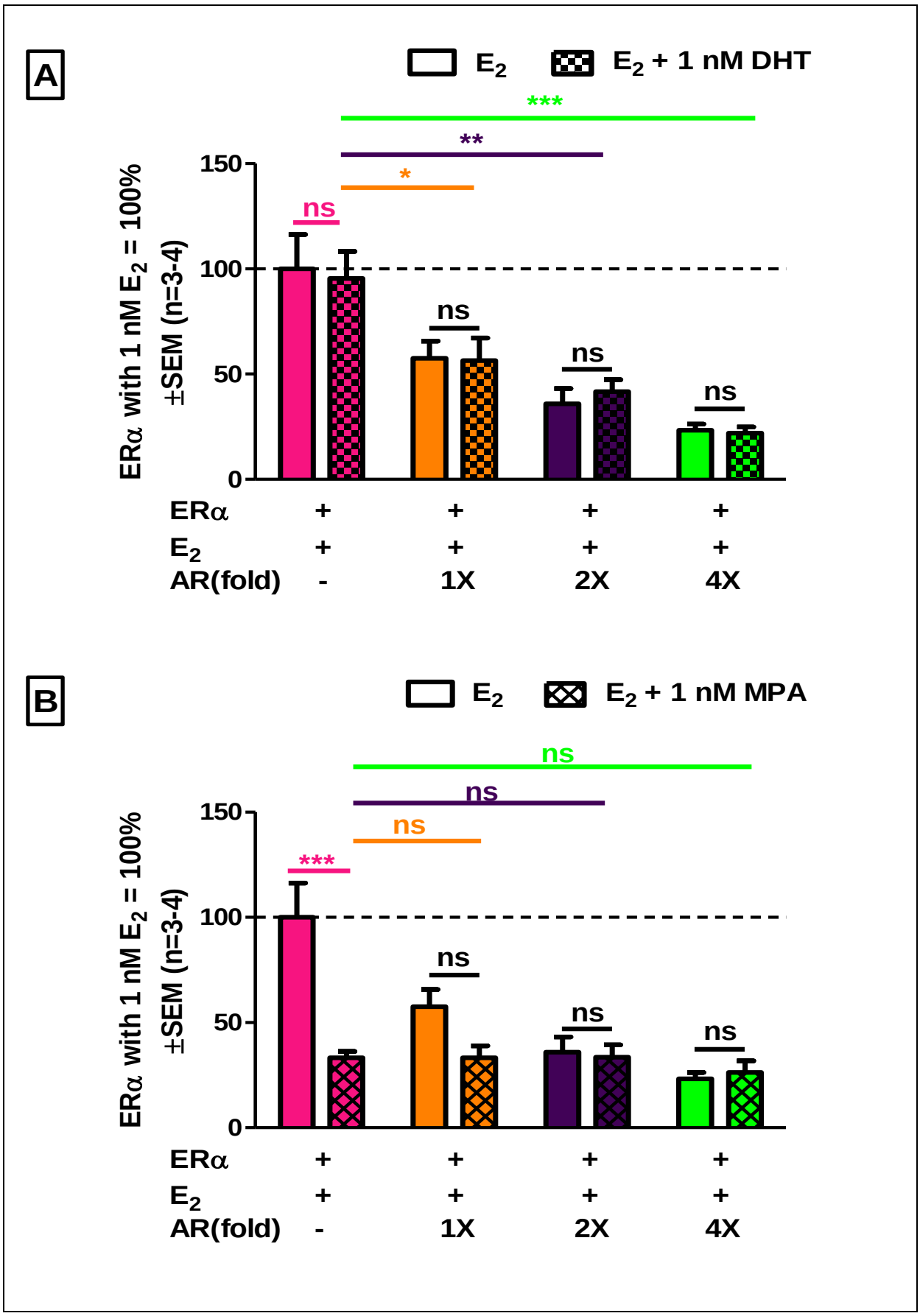


Figure 3.5. The AR does not decrease the transactivation function of E_2-bound ERβ. Human MDA-MB-231 breast cancer cells were transfected as in figure 3.4. The luciferase values were normalised to the protein concentrations, and expressed as luciferase/protein (luc/prot). The fold induction at 1 nM E_2 via ERβ is shown in **(A)**, while in **(B)** the fold induction in (A) was set as 100%, with induction in the presence of various concentrations of AR calculated relative to that. The results indicate the average (± SEM) of at least three independent experiments, with each condition performed in triplicate. Statistical analysis was performed using the unpaired t-test (A) and one-way ANOVA analysis of variance with Bonferroni (compare all pairs of columns) (B) post-tests.

3.3. The AR ligands do not augment the effect of the unliganded AR

Although we established that the unliganded AR does not affect E_2-bound ERβ transcriptional activation, we next investigated whether the ligand-activated AR would modulate ERβ activity. This was based on the fact that Peters and co-workers showed that when the AR is activated by its natural ligand, DHT or the selective AR modulator, MPA, a concentration dependent decrease in ERα activity was observed as the AR levels were increased (Peters *et al.* 2009). We also used DHT and MPA for model validation, but included NET-A, a progestin previously reported to have similar potent androgenic activity as DHT and MPA (Africander *et al.* 2014).

The human MDA-MB-231 cells were transiently transfected with 2.5 ng pSG5-ERα and 100 ng vit-ERE-TATA-luciferase or 2.5 ng pSG5-ERβ and 50 ng vit-ERE-TATA-luciferase, in the absence or presence of increasing concentrations of pSVARo (2.5 ng, 5 ng, 10 ng). The cells were subsequently treated with 1 nM E_2 in the absence or presence of 1 nM DHT, MPA or NET-A for 36 hours. The results in figure 3.6A-C show that inhibition of ERα transactivation function by the AR is not augmented by the addition of AR ligand. Surprisingly, cells transfected with only ERα and treated with equimolar concentrations of E_2 and MPA or E_2 and NET-A, displayed a significant decrease of E_2 activity via ERα (figure 3.6B-C), suggesting that MPA and NET-A are ERα antagonists. None of the AR ligands displayed agonist activity via ERα (figure 3.6D).



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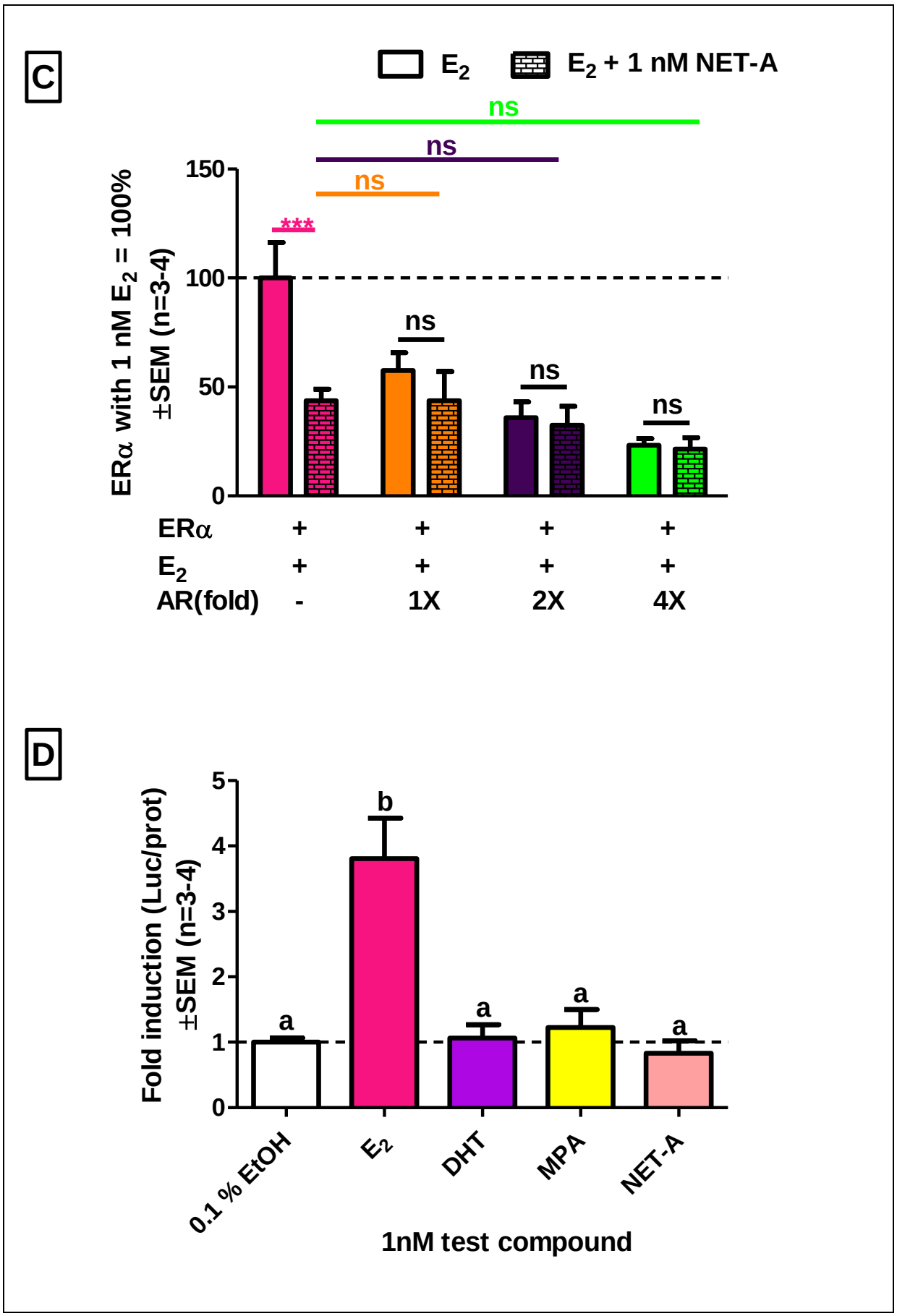

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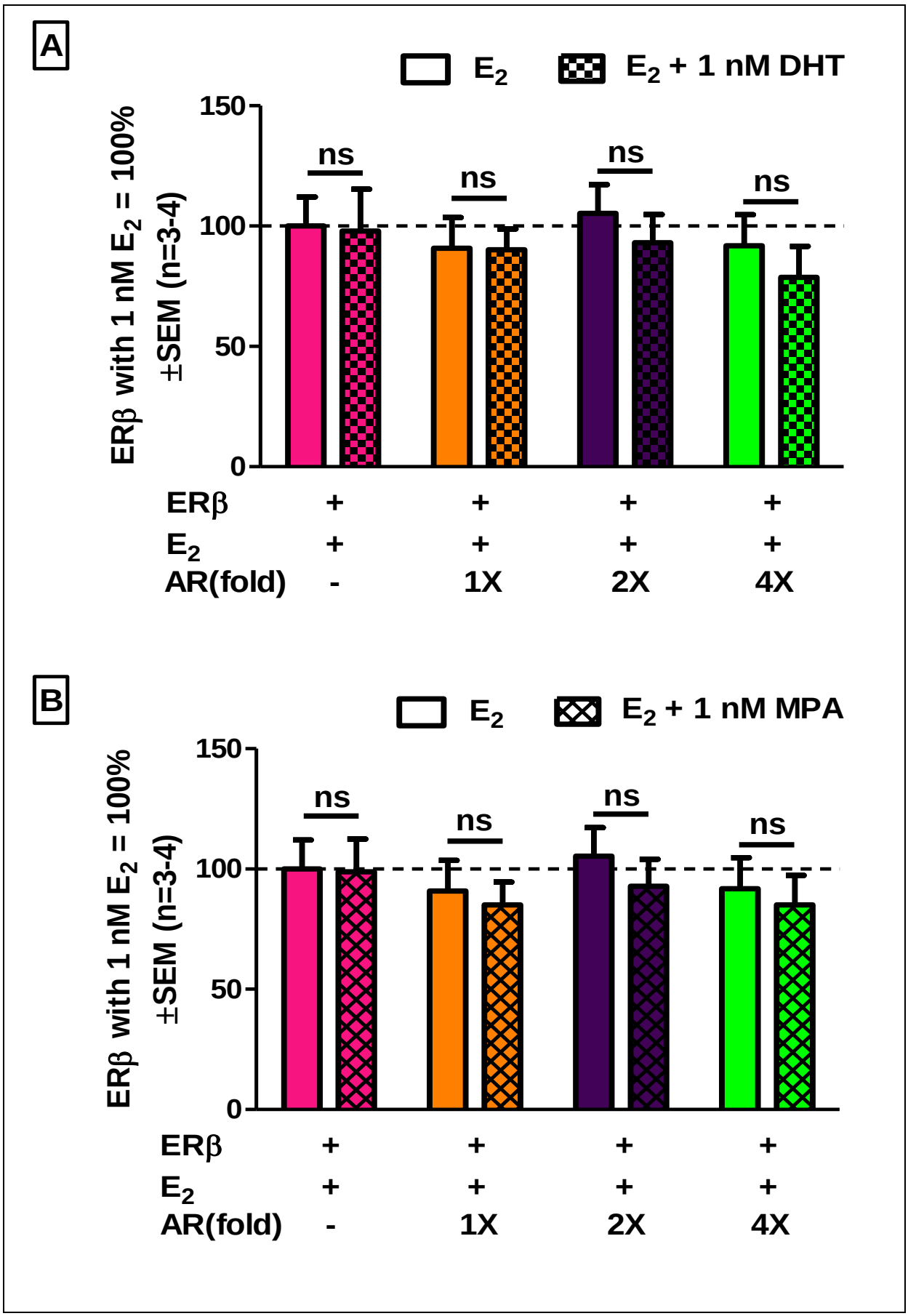
Figure 3.6. Inhibition of ERα transcriptional activation is not enhanced in the presence of AR ligands. Human MDA-MB-231 breast cancer cells were transiently transfected with pSG5-hERα and the vit-ERE-TATA-luciferase reporter construct, in the absence or presence of increasing concentrations of $pSVAR_O$ (1x, 2x or 4x molar excess), and where needed pGL2basic, using X-tremeGENE HP DNA transfection reagent according to the manufacturer's instructions. After 3 hours, cells were treated with 0.1% (v/v) EtOH (control) or 1 nM E_2 in the absence or presence of 1 nM DHT, MPA or NET-A for 36 hours. The luciferase values were normalised to protein concentrations, and expressed as luciferase/protein (luc/prot). The results indicate the average (± SEM) of at least three independent experiments, with each condition performed in triplicate. **(A -C)** The fold induction at 1 nM E_2 via ERα was set as 100%, and induction in the presence of various concentrations of liganded AR calculated relative to that. **(D)** The fold induction at 1 nM E_2, DHT, MPA and NET-A via ERα is shown. Statistical analysis was performed using Newman-Keuls (compare all pairs of columns) post-tests (A-C) and one-way ANOVA analysis of variance with Bonferroni (compare all pairs of columns) post-tests (D).

Similar to the results obtained for ERα, results in figure 3.7A-C indicate that the AR ligands do not modulate the effect of unliganded AR on the transactivation function of ERβ, and none of the AR ligands displayed agonist activity via ERβ (figure 3.7D). Interestingly, in contrast to the results seen for ERα (figure 3.6B-C), neither MPA nor NET-A had an inhibitory effect on the E_2-induced activity of ERβ (figure 3.7B-C). Taken together, these results indicate that the inhibition of ERα transactivation function by the AR is independent of AR ligand. Furthermore, the transactivation function of ERβ is not influenced by either the unliganded or liganded AR.



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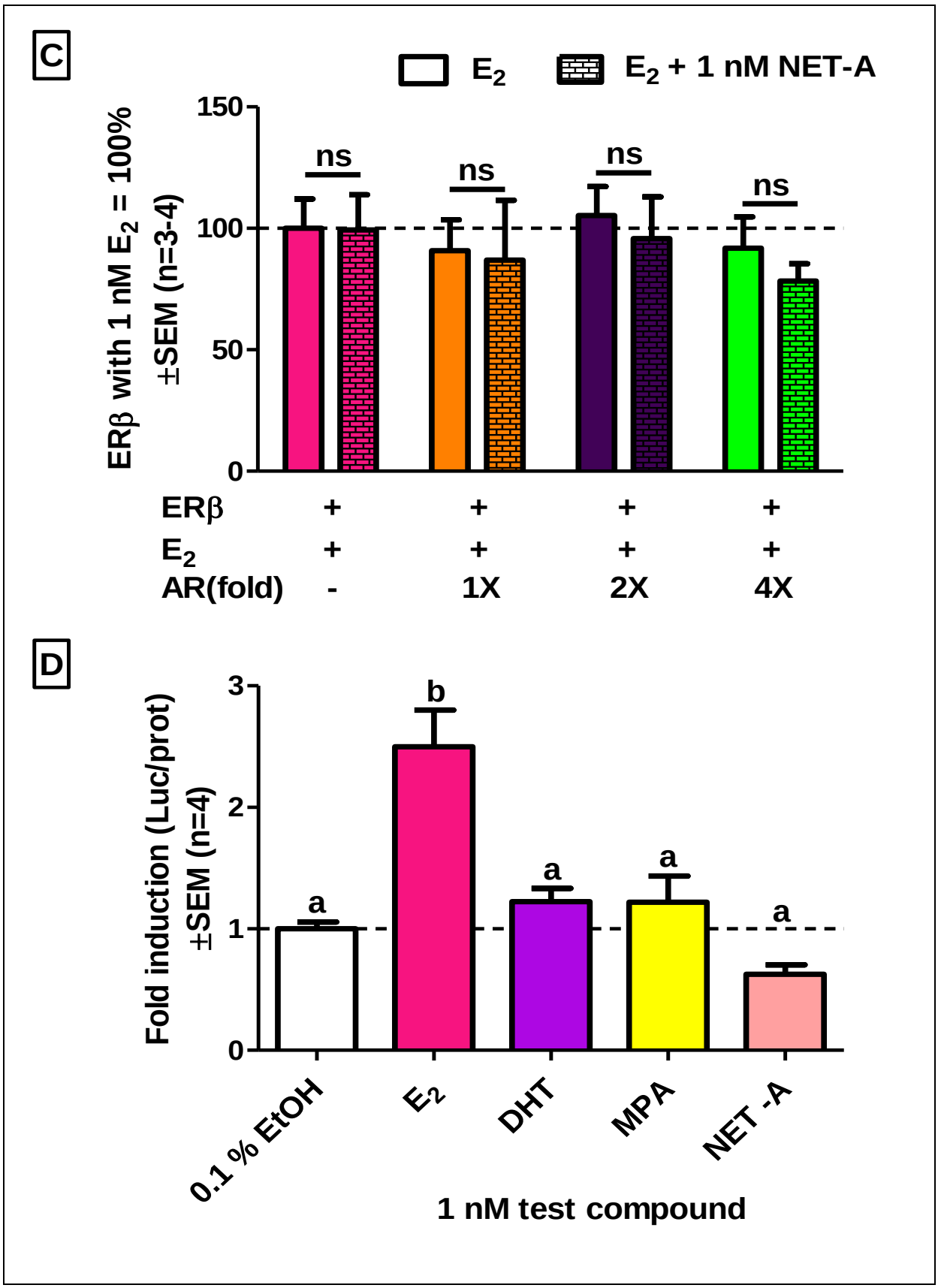

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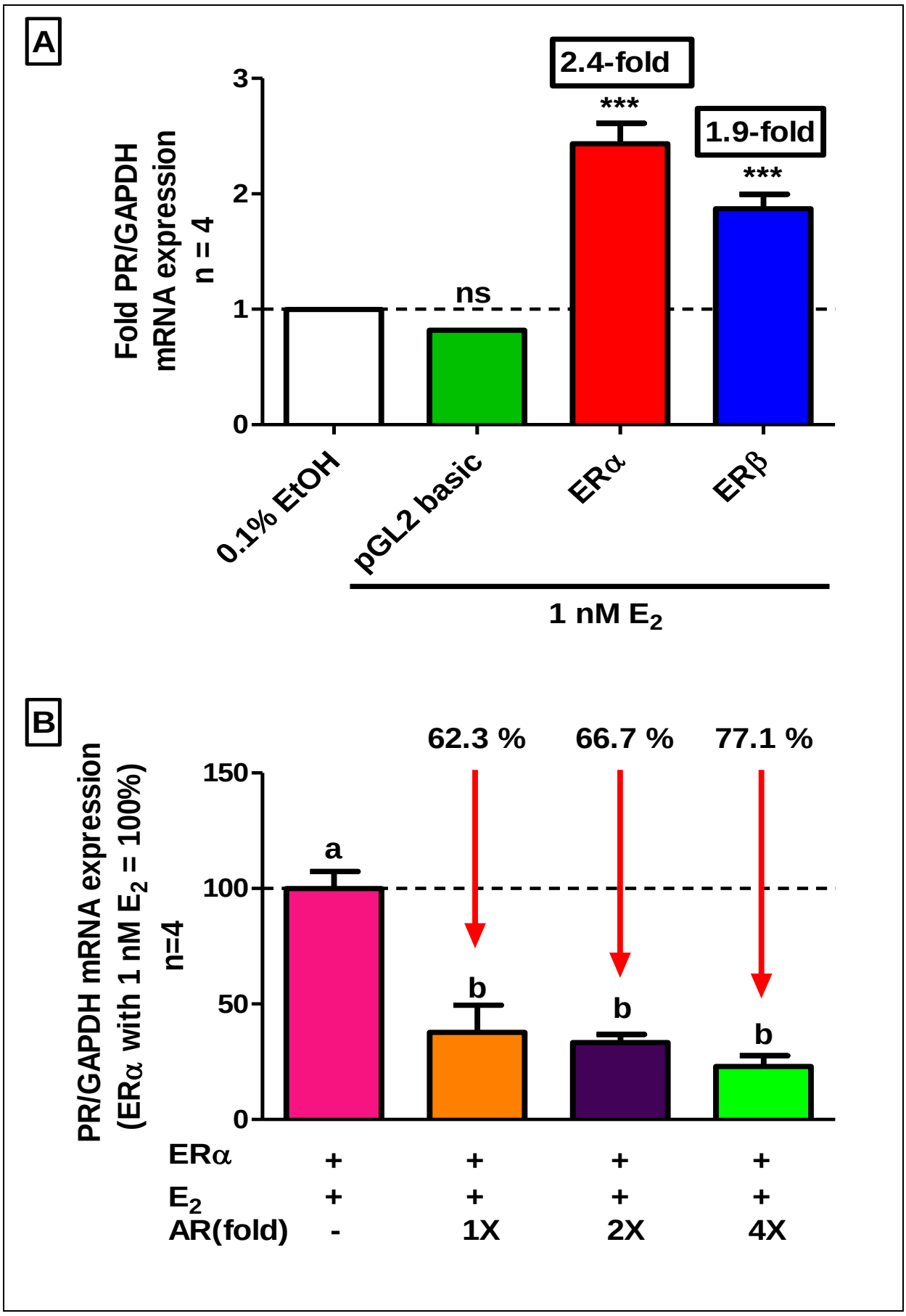
Figure 3.7. Ligand bound AR does not decrease the transcriptional activity of E_2 via ERβ. Human MDA-MB-231 breast cancer cells were transiently transfected with pSG5-hERβ and the vit-ERE-TATA-luciferase reporter construct, in the absence or presence of increasing concentrations of $pSVAR_O$ (1x, 2x or 4x molar excess), and where needed pGL2basic, using X-tremeGENE HP DNA transfection reagent according to the manufacturer's instructions. After 3 hours, cells were treated with 0.1% (v/v) EtOH (control) or 1 nM E_2 in the absence or presence of 1 nM DHT, MPA or NET-A for 36 hours. The luciferase values were normalised to protein concentrations, and expressed as luciferase/protein (luc/prot). The results indicate the average (± SEM) of at least three independent experiments, with each condition performed in triplicate. **(A -C)** The fold induction at 1 nM E_2 via ERβ was set as 100%, and induction in the presence of various concentrations of liganded AR calculated relative to that, and **(D)** The fold induction at 1 nM E_2, DHT, MPA and NET-A via ERβ. Statistical analysis was performed using two-way ANOVA analysis of variance with Bonferroni (compare all pairs of columns) post-tests (A-C) and one-way ANOVA analysis of variance with Bonferroni (compare all pairs of columns) post-tests (D).

3.4. AR abolishes the transcriptional activity of E_2 via both ER subtypes on an endogenous ER regulated gene in a breast cancer cell line

To investigate whether the results observed on the synthetic ERE-containing promoter could be mimicked on an endogenous ER regulated gene, the MDA-MB-21 breast cancer cell line was transiently transfected with 2.5 ng of either the ERα or the ERβ expression vector. The cells were treated with 1 nM E_2 for 12 hours, followed by qPCR analysis for the mRNA expression of the PR, a well-known ER regulated gene, which is used as a marker for functional ER signalling (Han *et al.* 2008, Sivik and Jansson 2012). The optimal time for PR gene expression in the presence of both ER subtypes was 12 hours (Addendum). The results in figure 3.8A show that E_2 upregulates PR mRNA expression in MDA-MB-231 cells transfected with either ERα (2.4-fold) or ERβ (1.9-fold). As a control to determine whether the E_2 response was indeed via the particular overexpressed ER subtypes, the cell line was transfected with 2.5 ng of the empty vector pGL2basic. The result in figure 3.8A shows that E_2 has no effect on PR gene expression

in the absence of the transfected ER subtypes, suggesting that the E_2-mediated upregulation of the PR gene is ER dependent.

Next we investigated how the presence of increasing AR concentrations would influence the E_2-induced upregulation of the PR gene, by transfecting the MDA-MB-21 breast cancer cell line with 2.5 ng of either the ERα or the ERβ expression vector in the absence or presence of 2.5, 5 or 10 ng of an AR expression vector. Note that, as we had shown that the addition of AR ligands in the presence of the AR did not influence ER transactivation on a synthetic ERE-containing promoter, we did not investigate PR gene expression in the presence of the liganded AR. The result in figure 3.8B shows that in the presence of transfected ERα, the unliganded AR appears to decrease the E_2-induced upregulation of the PR gene in a concentration dependent manner. However, the trend indicating that the decrease is concentration-dependent, was not statistically significant. Surprisingly, and in contrast to results on the synthetic ERE-containing promoter, the unliganded AR also appeared to decrease the E_2-induced upregulation of the PR gene in the presence of transfected ERβ (figure 3.8C).



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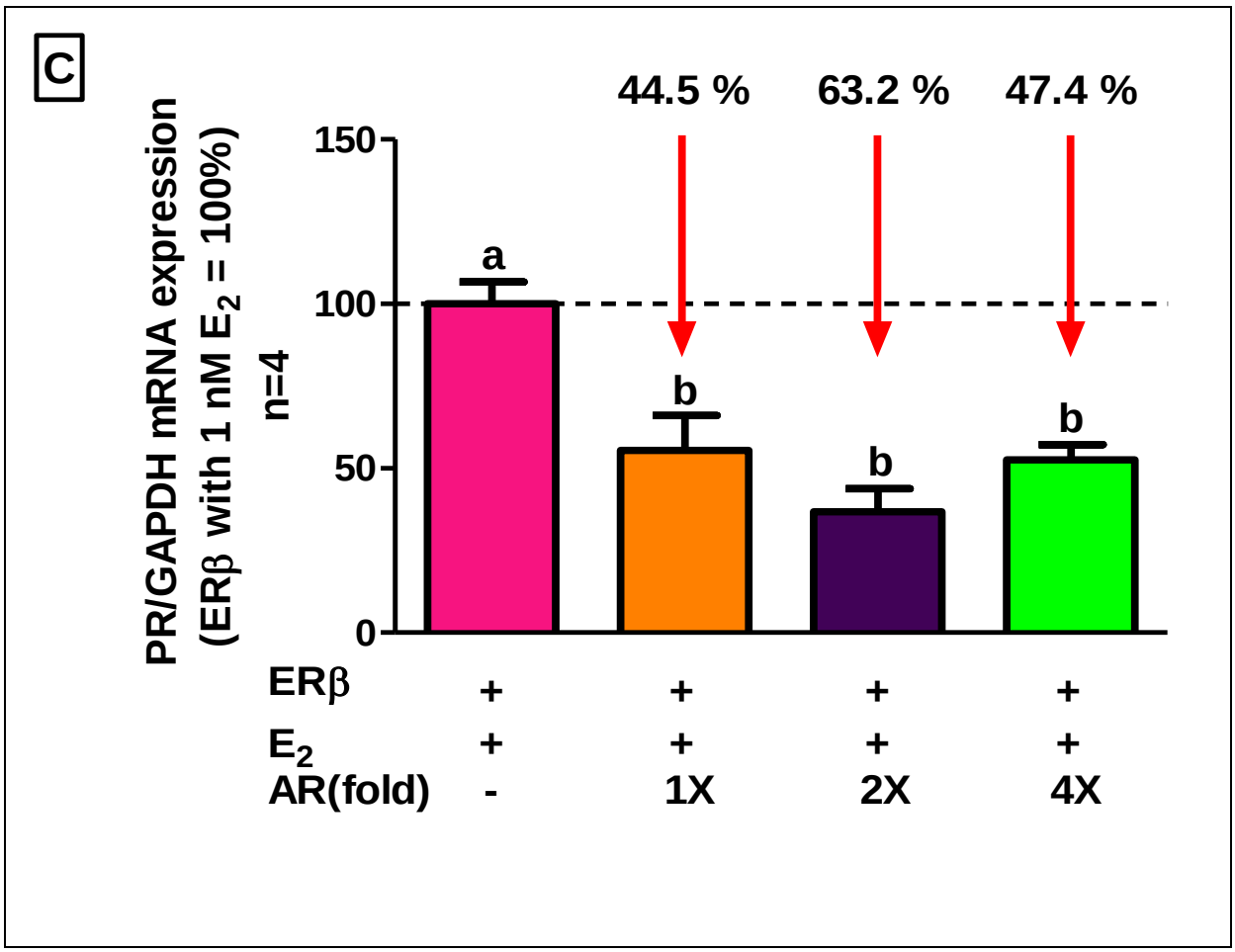

Figure 3.8. The E_2-induced expression of the PR gene in MDA-MB-231 breast cancer cells transfected with either ERα or ERβ is inhibited by the AR. Human MDA-MB-231 breast cancer cells were transiently transfected with pSG5-hERβ or pSG5-hERα in the absence or presence of increasing concentrations of $pSVAR_O$ (1x, 2x or 4x molar excess), and where needed the filler plasmid pGL2basic using X-tremeGENE HP DNA transfection reagent according to the manufacturer's instructions. After 24 hours, cells were treated with 0.1% (v/v) EtOH (control) or 1 nM E_2 for 12 hours. Total RNA was isolated, cDNA synthesized and mRNA expression levels of PR and GAPDH were determined by QPCR. GAPDH was used as the internal standard and the ratio of PR mRNA expression/GAPDH mRNA of samples treated with E_2 were calculated relative to EtOH. The fold induction is shown in (A), while this fold induction at 1 nM E_2 via ERα **(B)** or ERβ **(C)** was set as 100%, and mRNA expression in the presence of various concentrations of AR calculated relative to that**.** Results indicate the average (± SEM) of four independent experiments. Statistical analysis was performed using one-way ANOVA analysis of variance and Bonferroni (compare all pairs of columns) post-tests.

3.5. Androgen response element transactivation via ERα but not ERβ

It is well-known that the ER and AR activate transcription of target genes via binding to distinct response elements namely the ERE for ER (Kumar and Chambon 1988, Hyder *et al.* 1999, Ishida *et al.* 2010, Botelho *et al.* 2015), and the androgen response element (ARE) for AR (Mangelsdorf *et al.* 1995, Mitchell *et al.* 2000, Haendler *et al.* 2001, Matsumoto *et al.* 2013). However, recent evidence suggests that while ERα only binds to its consensus ERE, the AR can also bind to an ERE (Peters *et al.* 2009). Using promoter reporter assays, we thus next investigated whether either ERα or ERβ could transactivate via an ARE. MDA-MB-231 cells were transfected with 2.5 ng of either pSG5-ERα or pSG5-ERβ and 100 ng of the pTAT-GRE-E1b-luc promoter-reporter construct. As most steroid receptors, except the ER (Klock *et al.* 1987, Klein-Hitpass *et al.* 1988, Brosens *et al.* 2004), can bind the same DNA response element (Beato *et al.* 1989), the GRE can also serve as a response element for the AR, and is then referred to as an ARE. Results in figure 3.9A indicate that after treatment with 1 nM E_2 or DHT ERβ does not transactivate via an ARE ($p>0.05$). Considering that Peters *et al*. (2009) showed that ERα does not bind to an ARE, we were surprised when our results indicated significant transactivation ($p<0.001$) of an ARE-containing promoter-reporter construct via ERα (figure 3.9B). In addition, significant transactivation was also observed in the presence of 1 nM DHT (figure 3.9B).

As a control to determine whether the ARE-containing promoter-reporter construct is transcriptionally active, cells were also transfected with the pSVARo expression vector and the ARE-containing promoter-reporter construct, and treated with 1 nM DHT or E_2. As expected, induction (317-fold) was observed when cells transfected with the AR and an ARE were treated with 1 nM DHT (figure 3.9C), indicating that DHT and the ARE promoter-reporter construct are active in this cell line. No significant induction was observed with 1 nM E_2 indicating that E_2 has no agonist activity via the AR on an ARE.

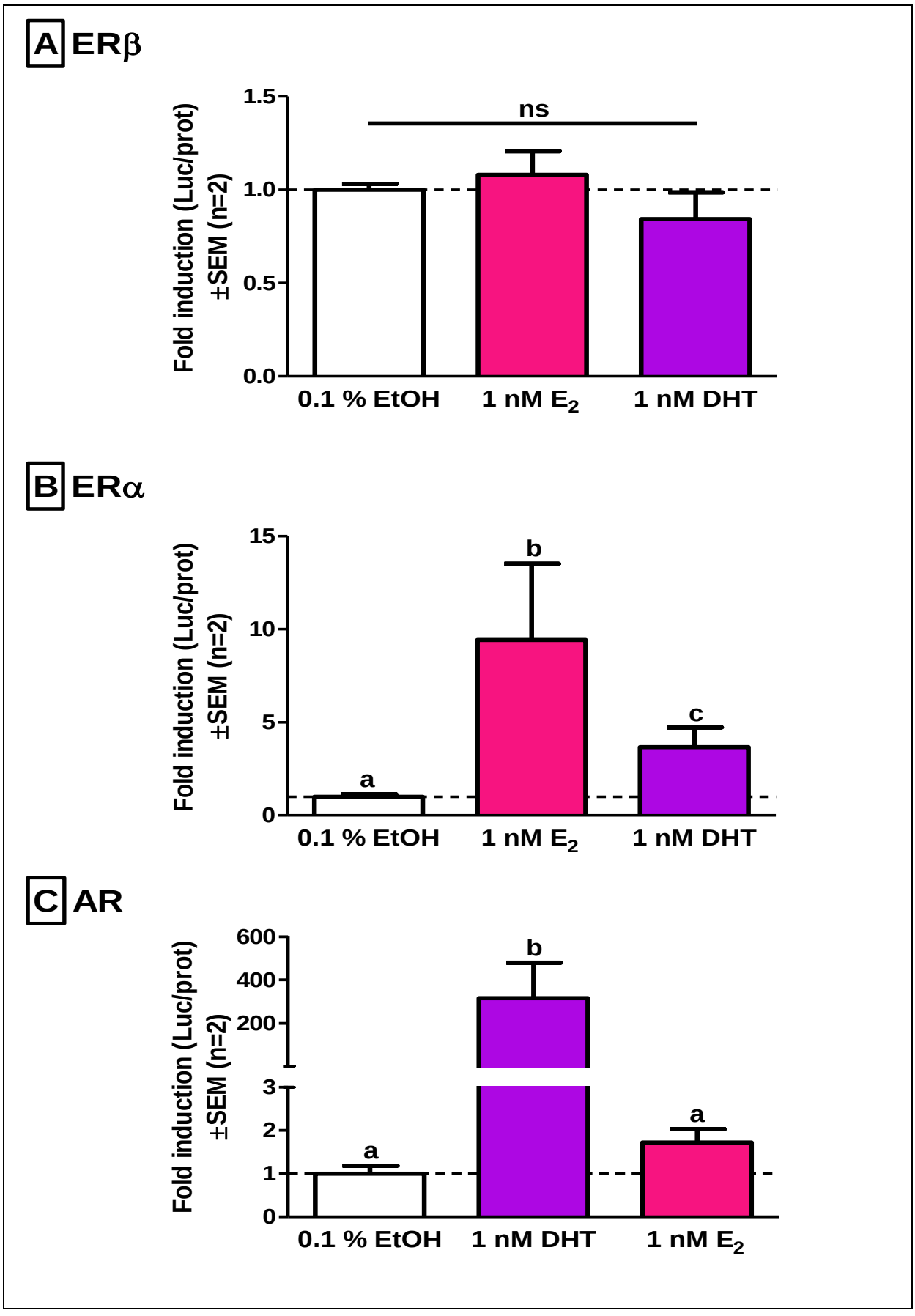

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Figure 3.9. Unlike ERα, ERβ does not transactivate via a synthetic ARE promoter-reporter construct. Human MDA-MB-231 breast cancer cells were transiently transfected with **(A)** pSG5-hERβ, **(B)** pSG5-hERα or **(C)** pSVAR$_O$ and a pTAT-GRE-E1b-luciferase containing promoter-reporter construct using X-tremeGENE HP DNA transfection reagent according to the manufacturer's instructions. After 3 hours, cells were treated for 36 hours with 0.1% (v/v) EtOH (control) or 1 nM E_2 or DHT. Luciferase values were normalised to the protein concentrations, and expressed as luciferase/protein (luc/prot). The fold induction via ERβ **(A),** ERα **(B)** and the AR **(C)** on an ARE is shown. The results indicate the average (± SEM) of two independent experiments, with each condition performed in triplicate. Statistical analysis was performed using one-way ANOVA analysis of variance and Bonferroni (compare all pairs of columns) post-tests.

3.6. E_2-induced upregulation of an endogenous ARE-containing gene can be mediated by both ERα and β

To investigate whether the results observed on the synthetic ARE-containing promoter could be mimicked on an endogenous ARE-containing promoter, the MDA-MB-21 breast cancer cell line was transiently transfected with 2.5 ng of either the ERα or the ERβ expression vector. The cells were treated with 1 nM E_2 or DHT for 12 hours, followed by qPCR analysis for the mRNA expression of the prostate specific antigen (PSA), which is a well-known ARE-containing gene. In contrast to the results in figure 3.9A and B, where we observed that unlike ERα, ERβ does not transactivate via a synthetic ARE, the results in figure 3.10A and B show that PSA mRNA expression was significantly upregulated in the presence of E_2 in cells transfected with either ERβ (6.0-fold) or ERα (2.6-fold). As expected, results from cells transfected with the AR and treated with 1 nM DHT showed significant upregulation (2.1-fold) of PSA gene expression (figure 3.10C).

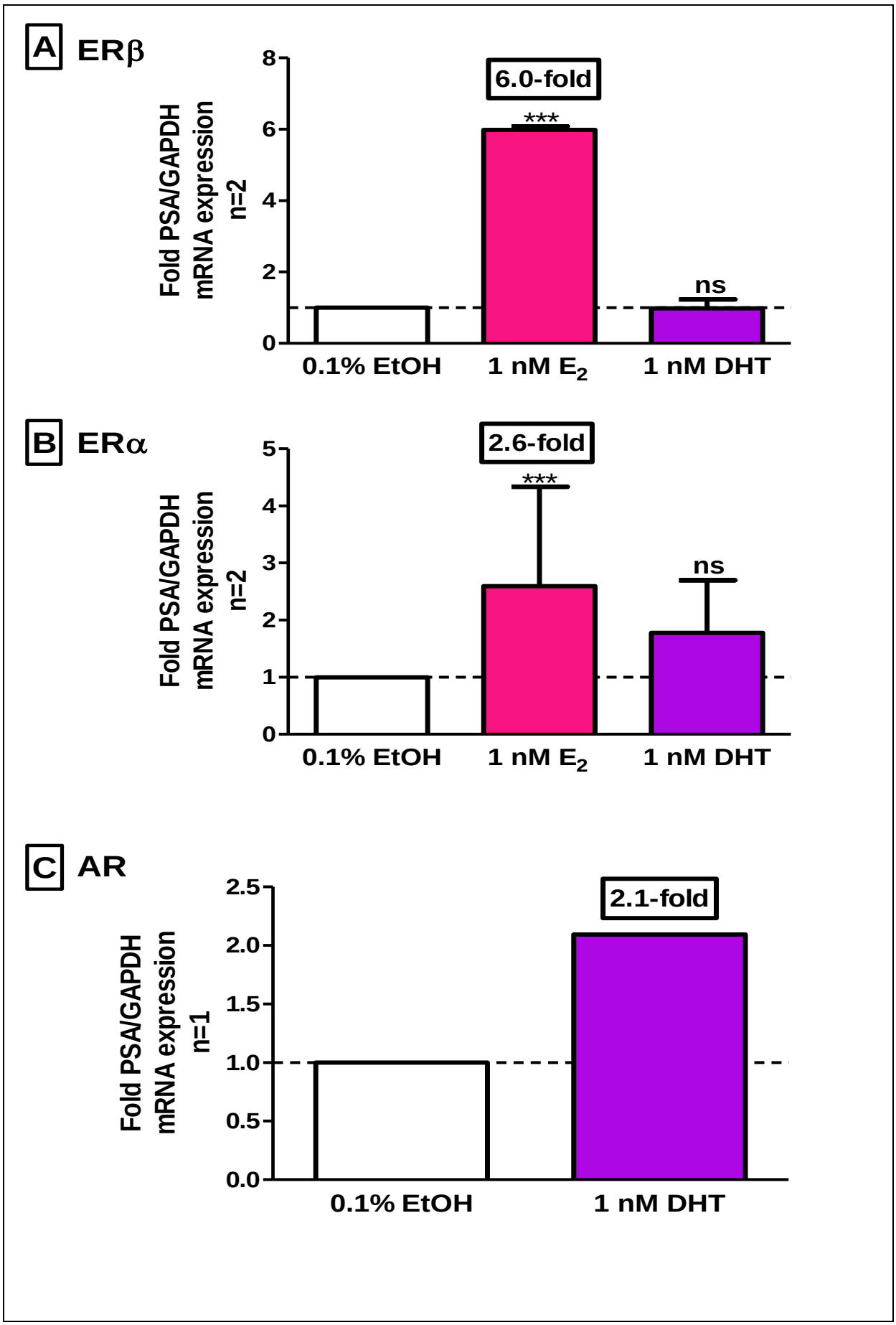

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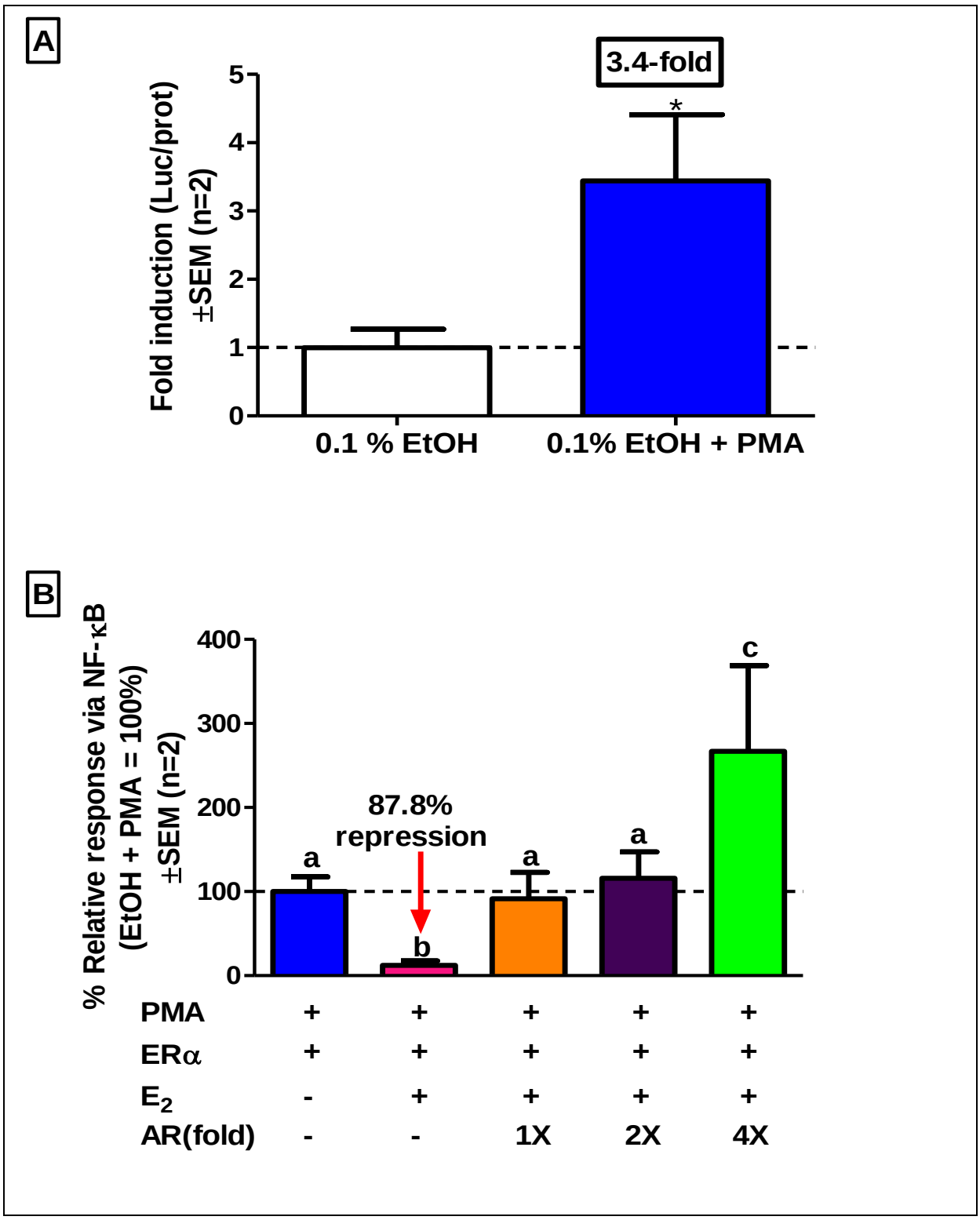
Figure 3.10. The E_2-induced mRNA expression of an AR regulated gene (PSA) via both ERα and ERβ. Human MDA-MB-231 breast cancer cells were transiently transfected with **(A)** pSG5-hERβ **(B)** pSG5-hERα or **(C)** $pSVAR_O$ using X-tremeGENE HP DNA transfection reagent according to the manufacturer's instructions and incubated for 24 hours. Cells were subsequently treated with 0.1% (v/v) EtOH (control) or 1 nM E_2 or DHT for 12 hours. Total RNA was isolated, cDNA synthesized and mRNA expression levels of PSA and GAPDH were determined by QPCR. GAPDH was used as the internal standard and the ratio of PSA mRNA expression/GAPDH mRNA of all samples were calculated relative to the sample treated with 0.1% (v/v) EtOH. Results show the PSA mRNA expression at 1 nM E_2 or DHT via **(A)** ERβ, or **(B)** ERα or **(C)** AR. For A and B results indicate the average (± SEM) of two independent experiments, while C is the result of a single experiment. Statistical analysis was performed using one-way ANOVA analysis of variance and Bonferroni (compare all pairs of columns) post-tests (A and B) and the unpaired t-test (C).

3.7. The AR inhibits the transrepression function of both ERα and ERβ

As the ER elicits its biological effects by either activating or repressing the transcription of target genes (Harrington *et al.* 2003), this study also examined the influence of the AR on the transrepression function of both ERα and ERβ. MDA-MB-231 cells were transiently transfected with 2.5 ng of either pSG5-ERα or pSG5-ERβ and 100 ng of a synthetic IL-6NFκB3-luciferase promoter-reporter construct, in the absence or presence of increasing concentrations of pSVARo. The cells were subsequently treated with 10 ng/ml PMA in the absence and presence of 1 nM E_2. As shown in figure 3.11A and C, stimulating the cells with PMA significantly induced an NFκB driven induction of 3.4-fold and 8.4-fold in the presence of ERα and ERβ, respectively. The PMA-induced response was then set to 100% and the E_2 response via ERα (figure 3.11B) or ERβ (figure 3.11D) calculated as a percentage relative to that. The E_2-mediated repression on the NFκB promoter, via ERα and ERβ, was 87.8% and 48.5%, respectively. While the addition of increasing concentrations of the AR inhibited the E_2-mediated repression on the NFκB promoter via ERα (figure 3.11B), E_2-mediated repression via ERβ was not affected by the AR (figure 3.11D).



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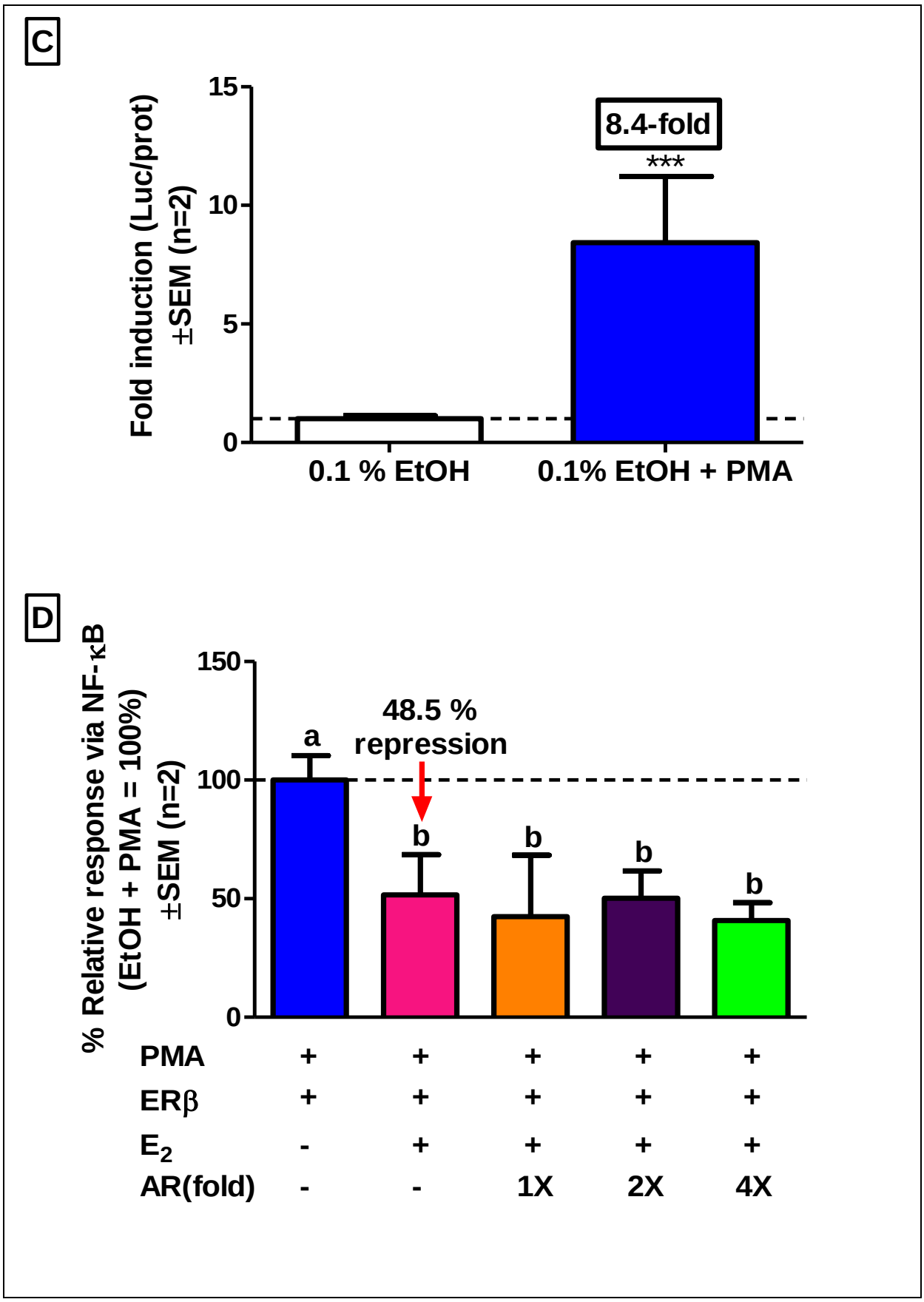

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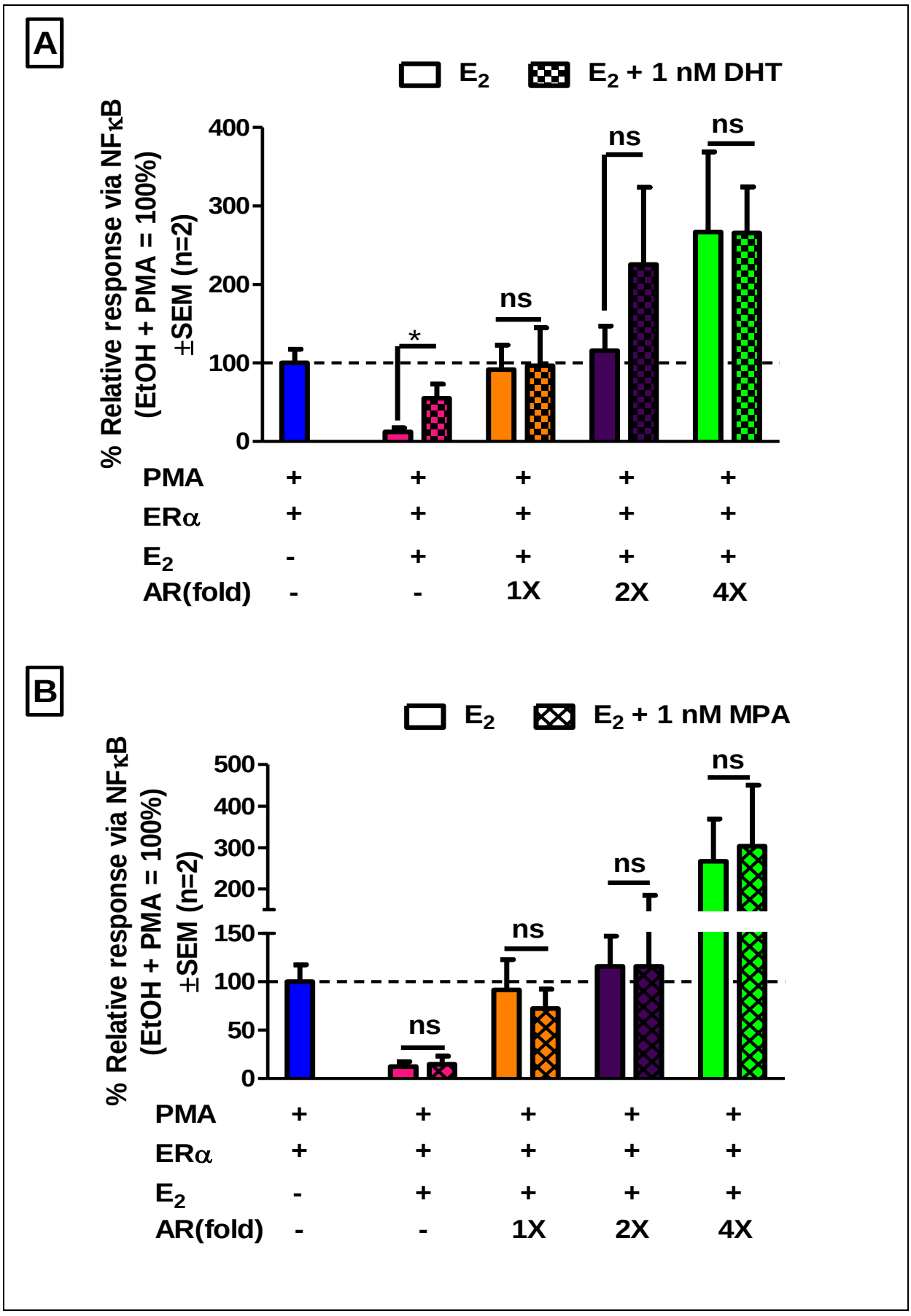
Figure 3.11. The AR inhibits the transrepression function of both ERα and ERβ. Human MDA-MB-231 breast cancer cells were transiently transfected with either **(A)** pSG5-hERα or **(B)** pSG5-hERβ and the IL-6NFκB3-luciferase containing promoter-reporter construct, in the absence or presence of increasing concentrations of $pSVAR_{O,}$ (1x, 2x or 4x molar excess), and where needed pGL2basic, using X-tremeGENE HP DNA transfection reagent according to the manufacturer's instructions. After 3 hours, cells were treated with 0.1% (v/v) EtOH (control) in the absence or presence of 10 ng/ml PMA **(A and C)** or 10 ng/ml PMA in the absence and presence of 1 nM E_2 **(B and D)** for 36 hours. The luciferase values were normalised to protein concentrations, and expressed as luciferase/protein (luc/prot). The results indicate the average (± SEM) of two independent experiments, with each condition performed in triplicate. **(A and C)** EtOH+PMA induction via the NF-κB-containing promoter construct calculated relative to the EtOH set as 1. **(B and D)** The PMA-induced response was set as 100% and the E_2-induced transrepression via ERα or ERβ, in the absence or presence of AR, was calculated relative to that. Statistical analysis was performed using unpaired t-test (A and C) and one-way ANOVA analysis of variance with Bonferroni (compare all pairs of columns) post-tests (B and D).

It was next investigated whether the presence of an AR ligand could modulate these effects. The human MDA-MB-231 cells were transfected as above and were subsequently treated with 1 nM E_2 in the absence or presence of 1 nM DHT, MPA or NET-A for 36 hours. Similar to results in figure 3.6 showing that the inhibition of ERα transactivation function by the AR is not enhanced by the presence of AR ligands, results in figure 3.12A-C indicate that AR ligands do not influence the AR-dependent inhibition of ERα transrepression function. Surprisingly, although the unliganded AR had no effect on the transrepression function of ERβ, results in figure 3.13A-C indicate that the ligand-bound AR inhibits the transrepression function of ERβ. This result is in contrast to the results in figure 3.7 showing that both the unliganded and liganded AR had no effect on the transactivation function of ERβ.



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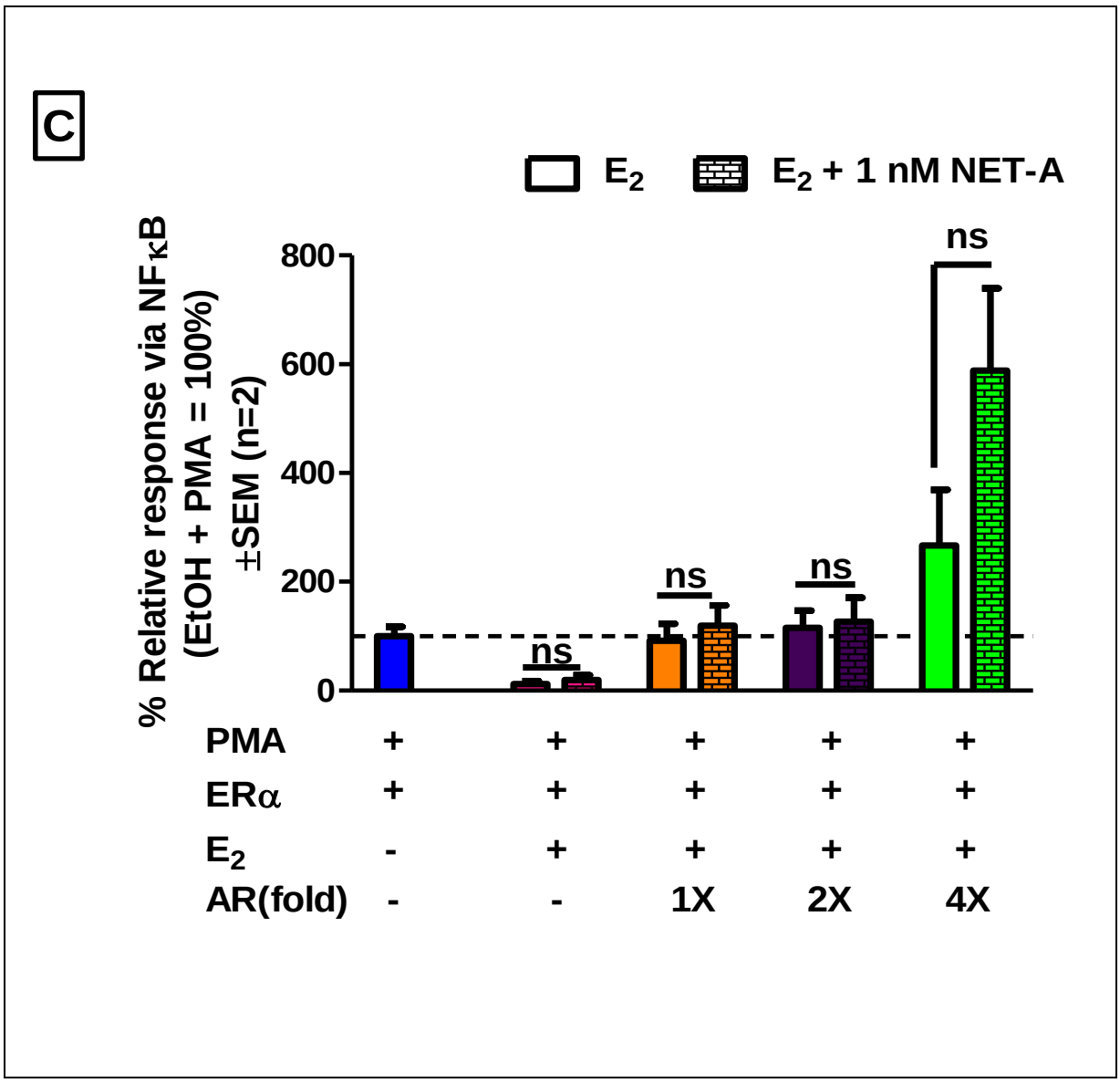
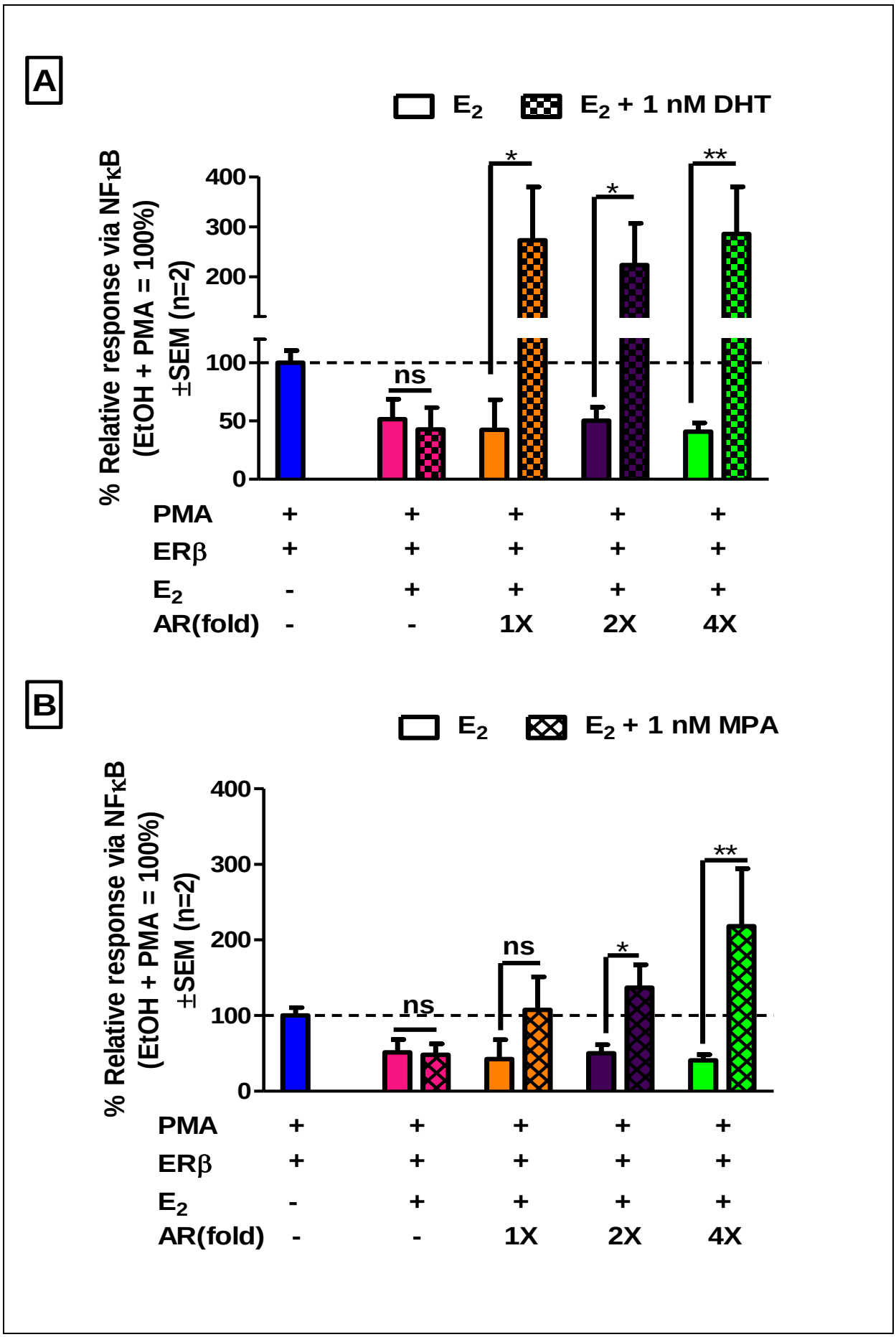


Figure 3.12. Inhibition of ERα transcriptional repression is not enhanced by the presence of AR ligands. Human MDA-MB-231 breast cancer cells were transiently transfected with pSG5-hERα and the IL-6NFκB3-luciferase containing promoter-reporter construct, in the absence or presence of increasing concentrations of pSVAR$_O$ (1x, 2x or 4x molar excess), and where needed pGL2basic, using X-tremeGENE HP DNA transfection reagent according to the manufacturer's instructions. After 3 hours, cells were treated with 10 ng/ml PMA and 1 nM E_2 in the absence or presence of 1 nM DHT, MPA or NET-A for 36 hours. The luciferase values were normalised to protein concentrations, and expressed as luciferase/protein (luc/prot). The results indicate the average (± SEM) of two independent experiments, with each condition performed in triplicate. The E_2-induced transrepression on the NFκB-containing promoter construct via ERα, in the absence or presence of AR, was expressed as a percentage calculated relative to EtOH + PMA set as a 100%. Statistical analysis was performed using one-way ANOVA analysis of variance with Bonferroni (compare all pairs of columns) post-tests as well as two-way ANOVA analysis of variance with Bonferroni (compare all pairs of columns) post-tests.



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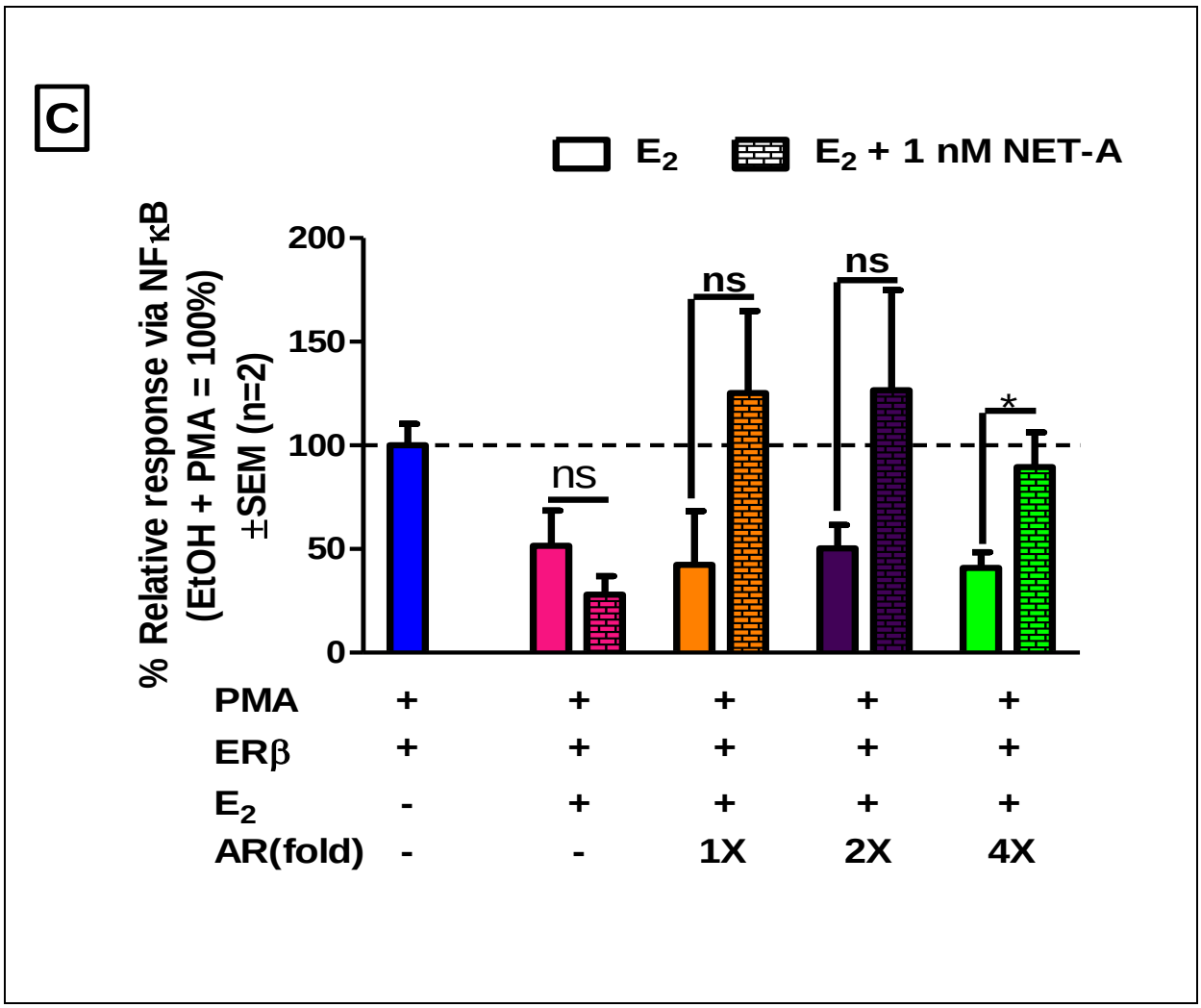

Figure 3.13. The ligand-bound AR inhibits the E_2-induced transrepression function of ERβ. Human MDA-MB-231 breast cancer cells were transiently transfected with pSG5-hERβ and the IL-6NFκB3-luciferase containing promoter-reporter construct, in the absence or presence of increasing concentrations of $pSVAR_O$ (1x, 2x or 4x molar excess), and where needed pGL2basic, using X-tremeGENE HP DNA transfection reagent according to the manufacturer's instructions. After 3 hours, cells were treated with 10 ng/ml PMA and 1 nM E_2 in the absence or presence of 1 nM DHT, MPA or NET-A for 36 hours. The luciferase values were normalised to protein concentrations, and expressed as luciferase/protein (luc/prot). The results indicate the average (± SEM) of two independent experiments, with each condition performed in triplicate. The E_2-induced transrepression on the NFκB-containing promoter construct via ERβ, in the absence or presence of AR, was expressed as a percentage calculated relative to EtOH + PMA set as a 100%. Statistical analysis was performed using one-way ANOVA analysis of variance with Bonferroni (compare all pairs of columns) post-tests as well as two-way ANOVA analysis of variance with Bonferroni (compare all pairs of columns) post-tests.

3.8. No change in proliferation was observed in cells transfected with ER

Cell proliferation assays were used to investigate E_2-induced proliferation of the MDA-MB-231 breast cancer cell line when transiently transfected with either ERα or ERβ, and how proliferation could be modulated by the presence of overexpressed AR. The MDA-MB-231 cell line was thus transfected with 2.5 ng of the expression vectors for either pSG5-ERα, pSG5-ERβ or the pGL2basic empty vector. The latter was used as a control to determine if E_2-induced cell proliferation was indeed via the overexpressed ER subtypes. As expected, no cell proliferation was observed for cells transfected with the empty vector (figure 3.14). Surprisingly, however, no E_2-induced cell proliferation was detected when cells were transfected with either ERα or ERβ (figure 3.14), and thus we could not investigate the effects of the AR on ER-mediated cell proliferation.

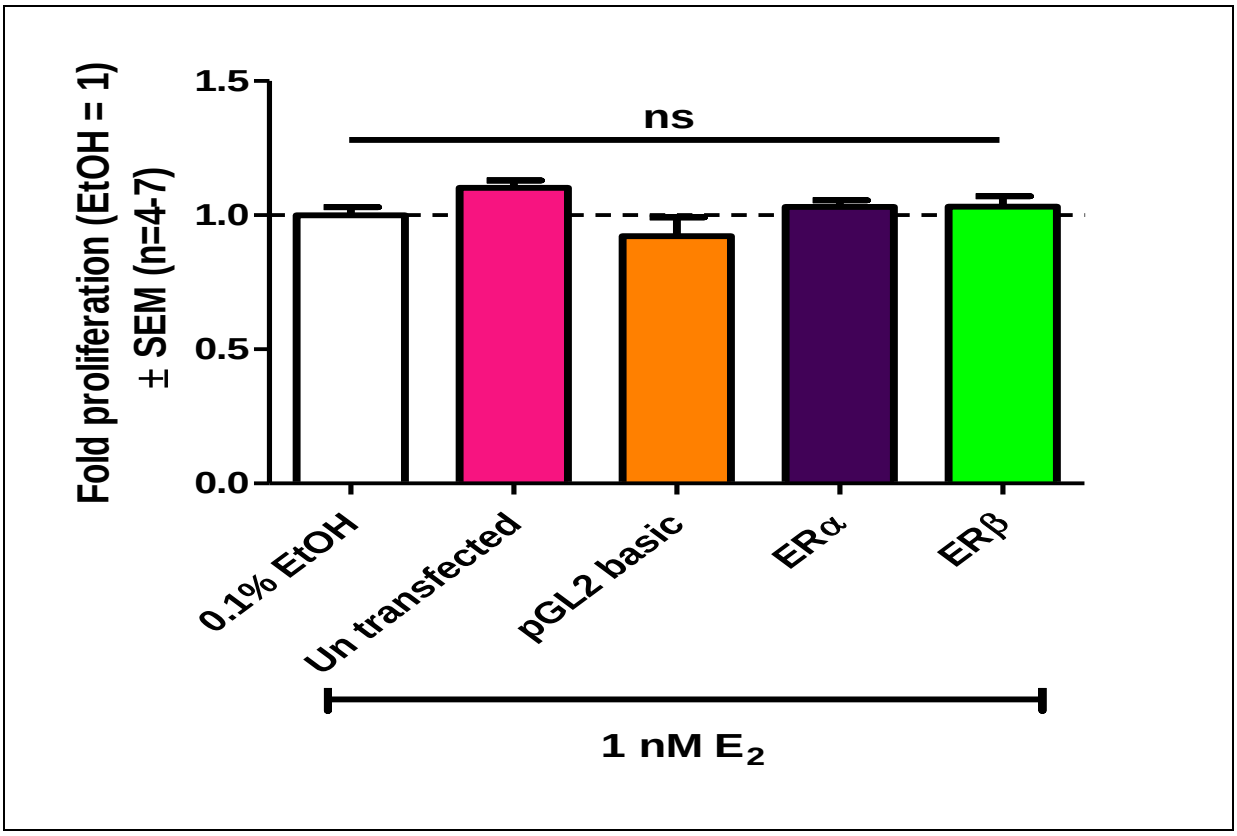

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Figure 3.14. Human MDA-MB-231 breast cancer cells were transiently transfected with pGL2basic (empty vector) or pSG5-hERα or pSG5-hERβ or using X-tremeGENE HP DNA transfection reagent according to the manufacturer's instructions. After 24 hours these cells, as well as untransfected MDA-MB-231 cells, were treated for 48 hours with 0.1% (v/v) EtOH (control) or 1 nM E_2. The results indicate the relative fold cell proliferation under various conditions with the value for cells treated with EtOH for each condition (control) set as 1. The average (± SEM) of at least four independent experiments, with each condition performed in triplicate is shown. Statistical analysis was performed using one-way ANOVA analysis of variance with Bonferroni (compare all pairs of columns) post-tests.

Chapter 4
Discussion

4.1. Introduction

Steroid receptors play an important role in the pathogenesis of breast cancer and understanding their clinical significance is a crucial aspect of breast cancer research (Welboren *et al.* 2007, Han 2014, Sabatier *et al.* 2014). The ER is the most common and well established breast cancer biomarker, with 75% of breast cancers being ER-positive. The ER exists as two subtypes, ERα and ERβ, which are transcription factors that modulate the expression of target genes (Couse and Korach 1999). The activated ERα stimulates breast cancer cell proliferation by regulating specific genes involved in the cell cycle (Frasor *et al.* 2003, Hartman *et al.* 2009), while ERβ has been shown to regulate a variety of target genes resulting in ERβ opposing the actions of ERα (Hartman *et al.* 2009). For example, E_2-induced ERα stimulates breast cancer cell proliferation by activating cyclin D1, an oncogene which regulates G1-S cell cycle progression, while ERβ is anti-proliferative as it inhibits cyclin D1 activation (Paech *et al.* 1997, Weatherman *et al.* 2001, Liu *et al.* 2002). Due to the distinct roles of ERα and ERβ in breast cancer, several studies have suggested that blocking the activity of ERα, but not ERβ, is an effective treatment option for breast cancer (Assikis and Craig 1997).

Although the role of the ER subtypes in breast cancer pathogenesis has been well-characterized, recent studies are also exploring the role of other steroid receptors (Peters *et al.* 2009, Need *et al.* 2012, Obr and Edwards 2012, Skor *et al.* 2013, Cochrane *et al.* 2014, Knutson and Lange 2014, Pistelli *et al.* 2014, Rechoum *et al.* 2014, Abduljabbar *et al.* 2015). For example, as the AR is expressed in 70% of breast cancer tumours (Birrell *et al.* 1998, Schippinger *et al.* 2006, Lin *et al.* 2009, Hu *et al.* 2011, Chottanapund *et al.* 2013), many clinical (Hu *et al.* 2011, Park *et al.* 2011, Pistelli *et al.* 2014) and molecular studies (Peters *et al.* 2009, Chottanapund *et al.* 2013, Cochrane *et al.* 2014, Rechoum *et al.* 2014) are investigating the role of the AR and androgens in breast cancer. Of particular interest is the fact that AR signalling is known to exert anti-estrogenic effects (Dauvois *et al.* 1991, Ando *et al.* 2002) suggesting crosstalk between the

ER and AR signalling pathways. Indeed, the study by Peters and co-workers showed that both the unliganded and liganded AR has the ability to inhibit the activity of ERα (Peters *et al.* 2009). In agreement with this, two other studies have also reported interplay between ERα and AR transcriptional pathways (Need *et al.* 2012, Rechoum *et al.* 2014). Whether crosstalk also exists between the AR and ERβ in breast cancer cells is not well-studied and thus formed the basis of this thesis. In chapter 3, it was thus investigated whether the unliganded and/or ligand-bound AR, could modulate the transcriptional activity (transactivation and transrepression) of ERβ in the human MDA-MB-231 breast cancer epithelial cell line.

4.2. Validating the model system

In the first part of this thesis, using western blot analysis (figure 3.1), previous reports in the literature indicating that the human MDA-MB-231 breast cancer cell line does not express the ER subtypes and the AR was confirmed (Horwitz *et al.* 1978, Lazennec and Katzenellenbogen 1999, Leo *et al.* 2004, Buchanan *et al.* 2005, Abduljabbar *et al.* 2015). This result, together with previous reports that the MDA-MB-231 cells do not express the PR, but express the GR and the MR, suggests that the human MDA-MB-231 cell line is an appropriate model cell line for comparing the activities of specific transiently transfected steroid hormone receptors.

Next, the experimental conditions used in the study by Peters *et al*. (2009) were mimicked, and in agreement with their results, the current study showed that the unliganded AR (figure 3.2B) inhibits the transcriptional activation of ERα (figure 3.2B). However, Peters and co-workers showed that the decrease in ERα transcriptional activation increased as the amount of liganded AR, activated by either the natural androgen DHT or the selective AR modulator MPA (MacLaughlin and Richardson 1979, Teulings *et al.* 1980, Birrell *et al.* 1998), was increased. The current study, on the other hand, showed that a concentration-dependent decrease in the transcriptional activation of ERα was observed in the presence of the unliganded AR, and that

the AR ligand (DHT, MPA or NET-A (a progestin with androgenic activity (Africander *et al.* 2014)), did not increase this effect (figures 3.2B; figures 3.6A-C). Furthermore, consistent with the result of Peters *et al*. (2009), the western blot analysis in figure 3.3 clearly indicates that the inhibition of ERα transcriptional activation by the AR was not due to the AR decreasing ERα protein levels.

Furthermore, our results show that MPA and NET-A, but not DHT antagonize the E_2-induced agonist activity for transactivation via ERα (figure 3.6B and C). It is known that progestins that exert androgenic properties, like MPA, are anti-estrogenic (Clarke and Sutherland 1990, Dauvois *et al.* 1991, Ando *et al.* 2002) via a mechanism that does not involve ER binding (Leung *et al.* 1981, Vignon *et al.* 1983, Sutherland *et al.* 1988). For example, it has been shown that MPA decreases intracellular E_2 levels as a result of it enhancing 17β-HSD2 activity, which causes the conversion of E_2 to E_1 (Dauvois *et al.* 1991, Pasqualini 2003). Considering that NET-A, like MPA, has potent androgenic properties (Africander *et al.* 2014), and that 17β-HSD type 1 and type 2 are expressed in MDA-MB-231 cells (Day *et al.* 2006, Newman *et al.* 2006), it is likely that the anti-estrogenic activity seen by both MPA and NET-A is due to these ligands increasing 17β-HSD2 expression and activity.

4.3. Effects on synthetic promoter-reporter genes are not always reflected on endogenous genes

Having validated the model system used by Peters *et al*. (2009) for ERα, we next used a similar model system to investigate the effect of the AR on the transactivation function of ERβ. Results in figure 3.5B showed that when MDA-MB-231 cells were co-transfected with the ERβ cDNA expression vector and increasing concentrations of the AR expression vector, the unliganded AR did not affect the E_2-induced transcriptional activation of ERβ. These findings are consistent with a previous study in CV-1 monkey kidney cells showing that co-expression of

the liganded AR with ERβ did not cause a significant decrease in ERβ transactivation (Panet-Raymond *et al.* 2000). Furthermore, our investigations in the presence of AR ligands (DHT, MPA, NET-A) showed that, like the unliganded AR, ligand-bound AR has no effect on the transactivation function of ERβ on a simple ERE (figure 3.7). Interestingly, using a yeast two-hybrid assay, Panet-Raymond and co-workers showed no interaction between the AR and ERβ, but like Peters *et al.* (2009) showed an interaction between the AR and ERα (Panet-Raymond *et al.* 2000, Peters *et al.* 2009). Interestingly, these observations occurred in the presence of either unliganded or liganded receptors. Several molecular mechanisms have been proposed on how the AR suppresses the transactivation function of ERα (Fioretti *et al.* 2014). For example, the AR and ERα have been shown to directly interact with each other thus preventing the latter from binding to EREs and as it has also been shown that the AR can bind to both synthetic and endogenous EREs (Peters *et al.* 2009, Fioretti *et al.* 2014), it can compete with ERα for binding to the EREs.

We next determined the effects of increasing amounts of unliganded AR on the mRNA expression of the endogenous PR gene. The PR is a well-known ER regulated gene, and its expression in breast cancer cells is used as a marker for functional ER signalling (Clarke *et al.* 1994, Yang *et al.* 2001, Rickard *et al.* 2002, Han *et al.* 2008). Furthermore, it has previously been shown that E_2-activated ERβ, like ERα, enhances the expression of the endogenous PR gene in the uterine and bone cells (Schultz *et al.* 2005). Surprisingly, although we showed that the AR, both unliganded and liganded, had no effect on ERβ-mediated transactivation in an ERE-reporter assay (figure 3.5B and 3.7A-C), the results in figure 3.8C showed that the unliganded AR downregulates the ERβ-mediated regulation of the endogenous PR gene. In contrast, the ability of the unliganded AR to inhibit ERα-mediated transactivation on the synthetic promoter-reporter gene (figure 3.2B) was replicated on the endogenous PR gene (figure 3.8B) with a similar percentage reduction observed at the higher AR concentrations (2- and 4-fold). These results indicate that although effects seen on a synthetic promoter-reporter

can often be reproduced on an endogenous gene, this is not always the case. Effects on the synthetic promoter are likely due to the AR and ERα competing for binding to ERE, as previously reported by Peters *et al*. (2009). However, considering that the AR inhibits the endogenous PR gene in the presence of either ERα or ERβ, it may be that effects on the endogenous promoter is more complex than effects on a simple synthetic ERE-promoter.

Although the general mechanism for transactivation is via the interaction of the activated ER with EREs in target genes, the human PR does not contain a palindromic ERE (Misrahi *et al.* 1988, Kastner *et al.* 1990, Schultz *et al.* 2005). Instead, it contains an ERE half-site and two specificity protein (Sp1) sites, all of which were previously shown to be needed for E_2-mediated activation of the PR gene (Schultz *et al.* 2003, Petz *et al.* 2004). Thus it may mean that ERα- and ERβ-mediated activation of the endogenous PR gene by E_2, requires both the ERE half site and the Sp1 sites. The possibility that the AR competes with either ERα or ERβ for the ERE half-site, can thus not be excluded. Furthermore, as the AR has been shown to repress Sp1-induced transcription (Curtin *et al.* 2001, Verras *et al.* 2007, Grosse *et al.* 2012), it may be possible for the AR to compete with both ERα and ERβ for tethering to Sp1, thereby inhibiting the activation of the PR gene. Taken together, we suggest a putative mechanism whereby the AR and ERα, but not ERβ, compete for binding to the synthetic ERE, while on the endogenous PR gene both ERα and ERβ compete with the AR for binding to either the ERE half-sites and/or for tethering to Sp1. However, further studies are needed to support this hypothesis.

4.4. E_2 upregulation of the endogenous ARE-containing PSA gene can be mediated by either ERα or ERβ

As mentioned previously, Peters and co-workers showed that the AR can bind to either an ARE or ERE, while ERα only binds to an ERE (Peters *et al.* 2009). However, we showed that E_2 via ERα could transactivate a simple ARE-containing promoter (figure 3.9B), as well as

upregulating the mRNA expression of the endogenous ARE-containing PSA gene in MDA-MB-231 cells (figure 3.10B). These results suggest, at least for the simple ARE in the current experimental system that ERα binds to the ARE. In addition, we show that E_2 upregulated PSA mRNA expression in the presence of overexpressed ERβ (figure 3.10A) but did not transactivate the simple ARE via ERβ (figure 3.9A), yet again, indicating that effects seen on a synthetic promoter cannot always be reproduced on an endogenous gene, and that regulation of an endogenous gene is more complex than regulation of a simple synthetic promoter. For the endogenous PSA gene, the possibility that ERα and ERβ could be interacting with *cis*-elements other than the ARE in the PSA promoter cannot be excluded. It is known that Sp1 binding sites are found in the PSA promoter (Sadar *et al.* 1999, Kim and Coetzee 2004), and as previously mentioned, it is known that ERα can tether to the Sp1 transcription factor to activate the transcription of the Sp1 containing PR gene (Petz *et al.* 2004, Schultz *et al.* 2005). Surprisingly, a 6.0-fold increase in the expression of the PSA gene was observed in the presence of ERβ and E_2 (figure 3.10A), while only a 2.1-fold was seen in the presence of the AR and DHT (figure 3.10C). However, it should be noted that the latter was the result of a single experiment, and should be repeated before any conclusions are made.

Interestingly the results in figure 3.9B showed that DHT also activates the simple ARE-containing promoter via ERα (a 3.7-fold increase). Although it appears that a similar activation (1.8-fold) of the endogenous PSA gene occurs (figure 3.10B), it was not statistically significant. To the best of our knowledge, there are no reports in the literature on the activation of ERα by DHT. However, at least one study has reported that DHT binds weakly to rat ERα and ERβ (Kuiper *et al.* 1997).

In summary, our results in MDA-MB-231 cells indicating that ERα can transactivate an ARE-containing promoter/gene are in contrast to the findings reported by (Peters *et al.* 2009) showing that ERα cannot bind to AREs in COS-1 and T47D cells. Taking into consideration that

receptors and their hormones behave differently in different cell lines (Birrell *et al.* 1998), the discrepancy in results could be due to the different techniques and/or cell lines used.

4.5. The unliganded AR impedes repression of transcription via ERα, but not ERβ, while the liganded AR impedes the transrepression function of both ERα and ERβ.

Most studies on the ER focus on the mechanism by which ERs regulate the transcription of E_2 regulated genes via direct binding to EREs. As described in Chapter 1 (Section 1.3.2.2), the ERs can also repress transcription of target genes by tethering to other DNA-bound transcription factors such as NFκB (Stein and Yang 1995, Galien and Garcia 1997). Thus, we next investigated whether the AR could modulate repression of transcription via the ER subtypes. In agreement with previously reported studies (Stein and Yang 1995, Galien and Garcia 1997, Visser *et al.* 2013), we showed that E_2 repressed the PMA-induced activation of a simple NFκB-containing promoter-reporter construct via both ERα and ERβ (figure 3.11B and D).

Similar to the results for transactivation of a simple ERE-containing promoter-reporter construct showing that the unliganded AR inhibits the transactivation function of ERα but not of ERβ (figure 3.2B and 3.5B), the results in figure 3.11B and 3.11D show that the unliganded AR inhibits the E_2-induced repression of NFκB activity via ERα but not via ERβ. To the best of our knowledge, this study is the first to show that the AR can modulate the transrepressive effects of E_2 via ERα. It is noteworthy that this modulation by the AR is not seen for ERβ. The inhibition noted in the case of ERα may be due to the unliganded and liganded AR interacting with ERα, physically preventing ERα from tethering to NFκB.

As seen for transactivation, the AR ligands do not modulate AR effects on ERα-mediated transrepression. Surprisingly, the results in figure 3.12A-C show that ligand-bound AR was able to inhibit the transrepression function of ERβ. Additional studies are needed to elucidate the mechanism behind this finding, as well as to evaluate its physiological impact. Considering that both the AR and ER subtypes can tether to NFκB leading to repression (Palvimo *et al.* 1996, Aarnisalo *et al.* 1998, McKay and Cidlowski 1999), it may be possible that the AR and ER subtypes are competing for binding to NFκB, thus the activated AR may prevent ERβ from causing transrepression thereby inhibiting the ER-mediated anti-inflammatory effects of E_2. It is known that inflammation plays an important role in breast cancer development and progression (Niu *et al.* 2012, Bhatelia *et al.* 2014, Suman *et al.*), thus our results showing that the AR reduces the anti-inflammatory effects of the ER may suggest that inflammation-induced breast cancer progression is promoted. However further studies are needed to assess the link between the ER, inflammation and breast cancer.

4.6. No change in proliferation was observed in cells transfected with ER

Transactivation and transrepression of ER target genes aid in the regulation of breast cancer cell proliferation (McKay and Cidlowski 1999, Beato and Klug 2000). Since the results from the current study showed that the unliganded AR could inhibit the transactivation and transrepression function of ERα, but not of ERβ, using simple ERE- and NFkB-containing promoter reporter constructs, we next evaluated the effects of the AR on E_2-stimulated proliferation of the human MDA-MB-231 breast cancer cell line. A MTT cell proliferation assay was thus performed in MDA-MB-231 cells transfected with either ERα or ERβ in the absence of the AR. As we did not detect E_2-induced proliferation in either the untransfected MDA-MB-231 cells or in those with overexpressed ERα or ERβ (figure 3.14), we could not evaluate the modulatory effects of the AR on cell proliferation.

Upon closer inspection of the literature, it appears that most studies employ the ER-positive MCF-7 breast cancer cell line (Han *et al.* 2008, Zhang *et al.* 2013) when investigating E_2-mediated cell proliferation. This is probably due to the fact that a number of studies have shown that cell proliferation is inhibited when ERα or ERβ is reintroduced into MDA-MB-231 cells (Garcia *et al.* 1992, Levenson and Jordan 1994, Lazennec *et al.* 2001, Moggs *et al.* 2005).

A possible explanation for the discrepancy in the results in the current study showing no proliferation versus the above-mentioned studies showing inhibition of proliferation, may be due to different experimental conditions used. For example, Garcia *et al*. (1992) treated MDA-MB-231 cells for 9 days and Lazennec *et al*. (2001) treated for 6 days, while in the current study cells were incubated for only 2 days. Furthermore, understanding why E_2 has an inhibitory effect on the proliferation of ER-negative cells when the ER is re-introduced, is a topic under investigation by a number of researchers (Garcia *et al.* 1992, Lazennec *et al.* 2001, Moggs *et al.* 2005).

4.7. Conclusions and Future Work

Several studies have shown that E_2 and ER play critical roles in breast cancer biology and their role in breast cancer has been extensively studied (Leygue *et al.* 1998, Speirs *et al.* 1999, Helguero *et al.* 2005, Yager and Davidson 2006, Zhao *et al.* 2008, Hartman *et al.* 2009). The ERα has been reported to promote cell growth and survival, while ERβ inhibits the ERα-mediated cell proliferation (Leygue *et al.* 1998, Dotzlaw *et al.* 1999, Speirs *et al.* 1999, Zhao *et al.* 2008). The role of other steroid receptors such as the AR in breast cancer is, however, an area of ongoing research, with emerging evidence suggesting that the AR is also involved in the regulation of breast cancer growth (Birrell *et al.* 1998, Brys 2000, Hartman *et al.* 2012, Shah *et al.* 2013, Lakis *et al.* 2014). For example, previous evidence suggests that the AR has the ability to inhibit the transactivation function of ERα, with not much known about the crosstalk

between the AR and the ERβ. Considering that the presence of either the AR or ERβ in ERα-positive breast cancers has been shown to be beneficial (Birrell *et al.* 1995, Szelei *et al.* 1997, Lanzino *et al.* 2005, Macedo *et al.* 2006, Williams *et al.* 2007, Hartman *et al.* 2009, Peters *et al.* 2009, Hickey *et al.* 2012), and that there is a lack of research investigating the interplay between the AR and ERβ in breast cancer, the aim of the current study was to elucidate the effects of the AR on the activity of ERβ.

The results presented in Chapter 3, showed that although the AR has no effect on the transactivation function of ERβ on a simple, synthetic ERE-containing promoter, it downregulates the ERβ-driven mRNA expression of the endogenous PR gene, a well-described ER-regulated gene. Considering that upregulation of this ER-regulated gene is associated with an increased risk of breast cancer (Fox *et al.* 2008, Han *et al.* 2008, Sivik and Jansson 2012) and evidence shows that ERβ acts like ERα when expressed in breast cancers lacking ERα, our results suggest that the AR may have a protective role in breast cancers expressing either ERβ or ERα. However, in inflammation-induced breast cancer the AR may not be protective considering results obtained in the current study for transrepression. Specifically, for transrepression studies, we show for the first time that both the unliganded- and ligand-bound AR inhibits the transrepression function of ERα, while only the ligand-bound AR was able to inhibit the transrepression function of ERβ. As it is known that inflammation plays a role in the development and progression of breast cancer (Niu *et al.* 2012, Bhatelia *et al.* 2014, Suman *et al.* 2015), our results suggest that the unliganded- or ligand-bound AR may promote breast cancer risk by reducing the anti-inflammatory effects of ERα, while the AR can only do so for ERβ when ligand bound. Furthermore, the current study also shows ERα-mediated, E_2-induced transactivation via a synthetic ARE as well as via an endogenous ARE-containing promoter, while the same is true for ERβ only via an endogenous ARE-containing promoter. It is interesting to note that ERβ seems to be upregulating the transcription of the endogenous PR and PSA genes via a mechanism involving tethering of ERβ to the Sp1 transcription factor.

While we cannot exclude that ERα also acts via tethering to Sp1, it appears that ERα activates transcription primarily via binding to EREs.

To the best of our knowledge this study is the first to show that the activated AR affects the transrepression function of both ERα and ERβ on a synthetic NFκB-containing promoter. A limitation of this study is that we did not investigate the influence of the AR on ER-mediated transrepression of any endogenous NFκB-containing promoters. This could be addressed on endogenous genes such as interleukin (IL)-6 or Regulated on Activation, Normal T-cell Expressed, and Secreted (RANTES) (Luboshits *et al.* 1999, Knüpfer and Preiß 2007), which are known to be involved in the development and progression of breast cancer and contain NFκB-binding sites within their promoters (Libermann and Baltimore 1990, Li and Bever 2001, Azenshtein *et al.* 2002). To investigate whether the presence of the AR inhibits the ability of the ER subtypes to tether to NFκB, as suggested in Section 4.5, co-immunoprecipitation (Co-IP) and/or fluorescence resonance energy transfer (FRET) assays may be used. Co-IP assays are used investigate protein-protein interactions *in vitro* (Fields and Song 1989), while the FRET assay allows the investigation of protein-protein interaction in living cells (Clegg 1995). A further limitation was that we did not plate into a 10 cm dish for transfections, therefore protein concentration was not the best normalization method.

Lastly, as no E_2-stimulated proliferation could be obtained in the ER-negative breast cancer cell line containing overexpressed ERα or ERβ, the effect of the AR on ER-mediated cell proliferation could not be investigated. Proliferation assays should thus be carried out in a cell line which is known to express both ER subtypes such as the MCF-7 or T47D breast cancer cell line. The role of the AR on ERα- or ERβ-mediated breast cancer cell proliferation can be determined in these cell lines by silencing the expression of the respective ER subtype using siRNA technology

To gain more insight into how the AR modulates ERβ-mediated regulation of the PR gene, chromatin immunoprecipitation (ChIP) assays can be used to firstly determine whether ERβ is recruited to *cis*-elements such as the ERE-half sites and/or Sp1 binding sites in the PR promoter. Secondly, ChIP can be used to determine whether the AR competes with ERβ for binding to the above-mentioned *cis*-regulatory elements as it has previously been shown that the AR can bind to the ERE and can tether to the Sp1 (Curtin *et al.* 2001, Verras *et al.* 2007, Peters *et al.* 2009, Grosse *et al.* 2012). The ChIP assay is considered a relatively inexpensive method to study the interaction between a protein and a specific DNA sequence *in vivo*. Furthermore, to establish whether the AR interacts with ERβ and the Sp1 transcription factor to form a complex on the promoter of the PR gene, sequential chromatin immunoprecipitation (re-ChIP) assays may be employed. The mechanism whereby ERα and ERβ upregulates the mRNA expression of the PSA gene, which contains AREs, Sp1 binding sites as well as other *cis* regulatory elements, can also be further investigated by ChIP assays. Besides studying the recruitment of the ER subtypes to the Sp1 *cis*-regulatory element, similar experiments as described above may be used to determine whether the ER subtypes interact with the Sp1 transcription factor on the PSA promoter.

To examine whether the Sp1 transcription factor is required for ERβ-mediated upregulation of PR gene expression, siRNA technology can be used to silence the expression of Sp1. In addition, it would be interesting to examine the effects of the AR on other ERβ-regulated endogenous genes such as cathepsin D (CTSD) (Behrens *et al.* 2007), as it has previously been shown that the AR inhibits the expression of the ER regulated PR gene, but not the CTSD gene (Peters *et al*. 2009). Lastly, although the use of a yeast two-hybrid assay has previously shown that the AR and ERβ do not interact with each other, it would be of interest to determine whether a direct association between these steroid receptors exists in our model system. The latter could be investigated using either Co-IP or FRET assays.

In summary, the results presented in this thesis showing that the AR inhibits the activity of ERα via a synthetic ERE-promoter as well as an endogenous ER-regulated gene and that of ERβ via an endogenous ER-regulated gene, may suggest that the presence of AR is also a good prognostic marker for breast cancer patients as previously reported for ERα by Peters *et al.* (2009). On the other hand, our novel results showing that the activated AR reduces the anti-inflammatory effects of the ER, may suggest that the AR promotes inflammatory-induced breast cancer. The precise physiological implications of these results remain to be determined. Finally, although the results from this study are preliminary and have certain limitations, it nonetheless highlights the fact that the role of the AR in breast cancer is a complex one. Our findings may thus aid in understanding of crosstalk between the ER and AR signalling pathways, and how it contributes to the growth and survival of breast cancer cells. Figure 4.1 shows the contribution of findings of this current study to the existing knowledge on the interplay between the AR and ERβ.

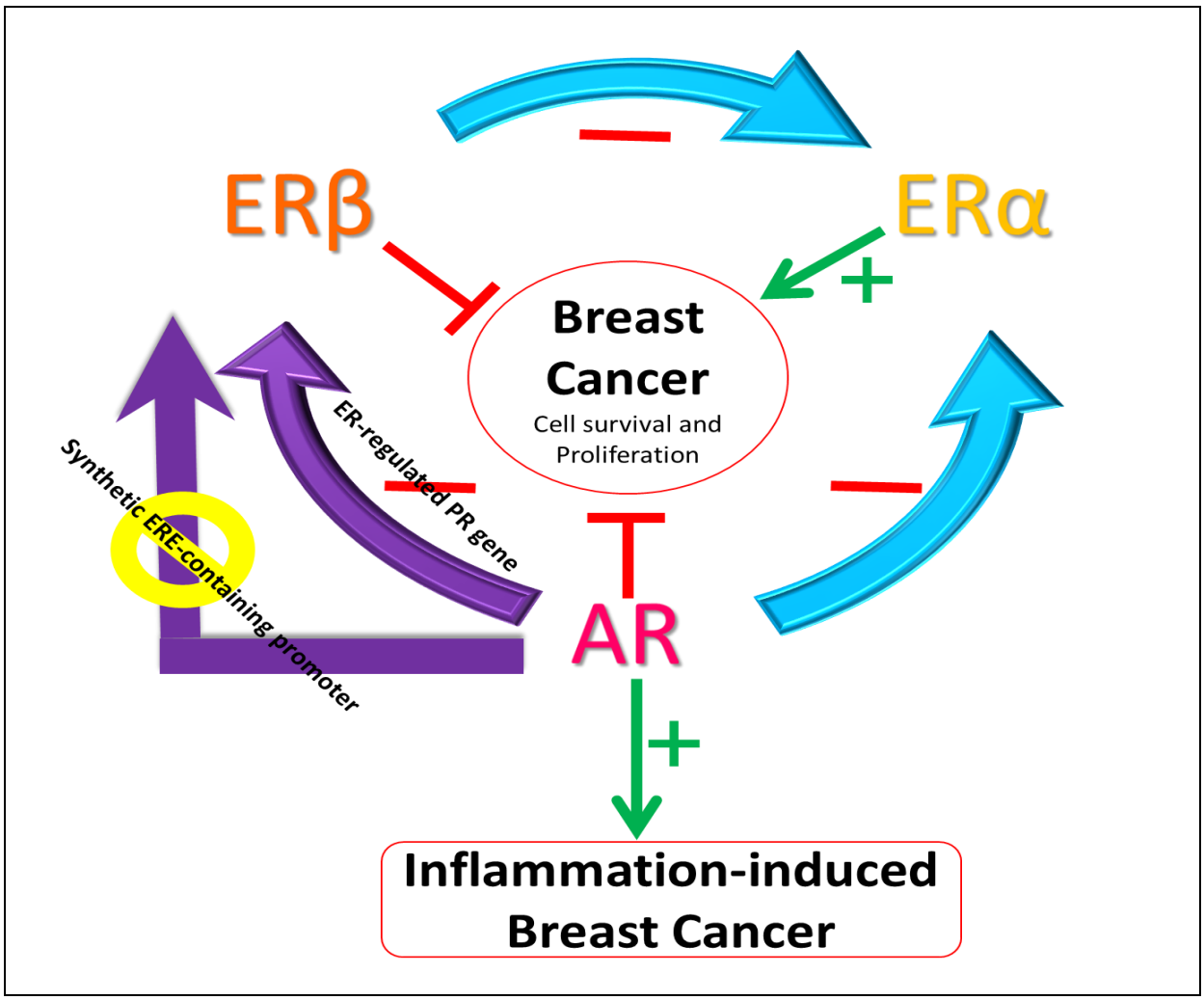

Figure 4.1. Interplay between the ER subtypes and the AR may lead to both positive and negative effects on breast cancer. ERα promotes cell survival and proliferation, while ERβ antagonizes ERα-mediated cell proliferation. The AR has been shown to have the ability to inhibit cell proliferation both directly and indirectly by suppressing the activity of ERα. The AR also has no effect on the activity of ERβ on synthetic ERE-containing promoter, while the AR inhibits the upregulation of the ER-regulated PR gene. Lastly the AR promotes inflammation-induced breast cancer by inhibiting the transrepression function of both ERα and ERβ. – and T denote inhibition of activity, + shows activation of activity, the yellow circle with a line inside indicates no effect on the activity, the blue arrows demonstrate that the effect is towards ERα while the purple arrow demonstrates that the effect is towards ERβ.

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ADDENDUM

Realtime quantitative polymerase chain reaction (qPCR)

Realtime quantitative PCR is a powerful method used for the measurement of gene expression (Celi *et al.* 1994). qPCR is based on the original PCR method that employs forward and reverse primer sequences that are specific for certain target genes. However, qPCR has been modified to involve the use of fluorescent dyes or probes (Pfaffl 2001, Huang *et al.* 2014, Navarro *et al.* 2015). Fluorescent dyes such as SYBR-green are used during qPCR as it binds to the minor groove of the double stranded DNA. The use of SYBR-green allows simultaneous PCR amplification, detection of the PCR product, quantification of the new double stranded DNA being formed and lastly verification of the PCR product using melt curve analysis (Morrison and Weiss 1998, Phillips *et al.* 2009). However, there are certain factors that should be considered when conducting qPCR such as making sure that the reference gene does not give any expression under the experimental conditions as well as using RNA that is of good quality (Bustin 2002, Udvardi *et al.* 2008).

To assess gene expression, high quality, intact RNA has to be isolated to ensure that experimental results obtained are reliable as poor quality RNA can compromise results. RNA quality was assessed by determination of the optical density (OD) at 260 nm (nucleic acids) and the 280 nm (proteins), followed by analysis on a 1% denaturing formaldehyde agarose gel. The latter is to analyse the quality and integrity of RNA isolated from the cells as the 260 nm OD reading can be compromised by the presence of genomic DNA (Sambrook *et al.* 1989). An OD 260/280 ratio between 1.9 and 2.1 indicates that the RNA is pure (Sambrook *et al.* 1989). As shown in figure A1, the intact total RNA isolated from the MDA-MB-231 cells showed sharp, clear 28S and 18S RNA bands. As the 28S RNA band is approximately twice the intensity of the 18S RNA band, it is a good indication that the RNA is intact (Krebs *et al.* 2009). If the 28S and 18S RNA bands appearance as a smear, it is an indication that the RNA is degraded, while genomic DNA does not migrate through the gel because of its size (Sambrook *et al.* 1989).

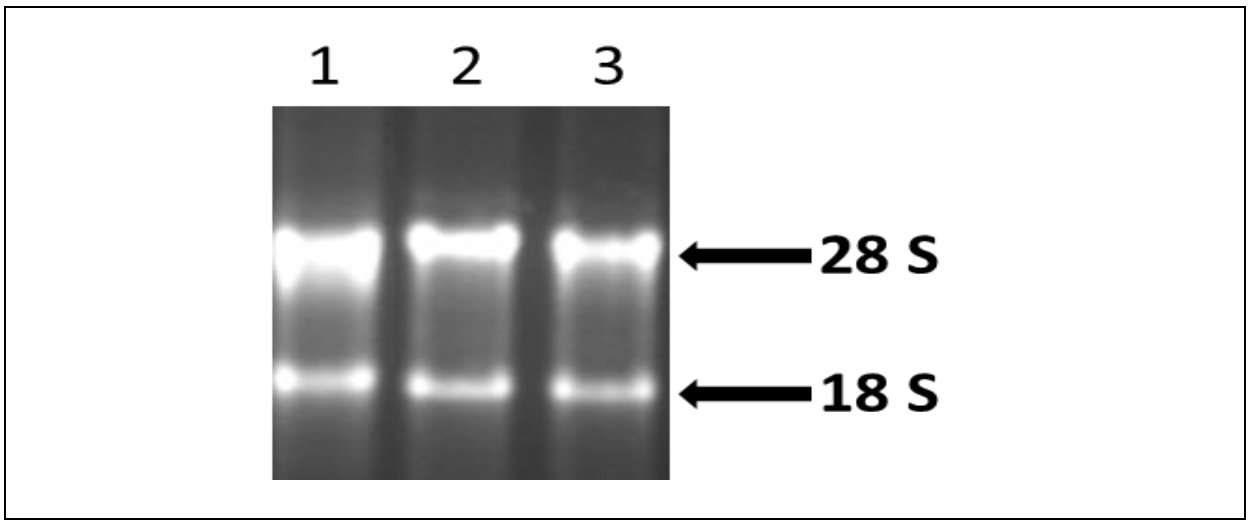


Figure A1. A representative 1% denaturing formaldehyde agarose gel showing the intact total RNA isolated from MDA-MB-231 cells. Total RNA was isolated from MDA-MB-231 cells transiently transfected with 2.5 ng pGL2basic, ERα or ERβ, as described in section 2.6 of Chapter 2. A total of 1 µg RNA was electrophoresed on a 1% agarose gel and visualised by ethidium bromide staining.

The intact RNA was then used to synthesize complementary DNA (cDNA) as described in section 2.6 of Chapter 2.

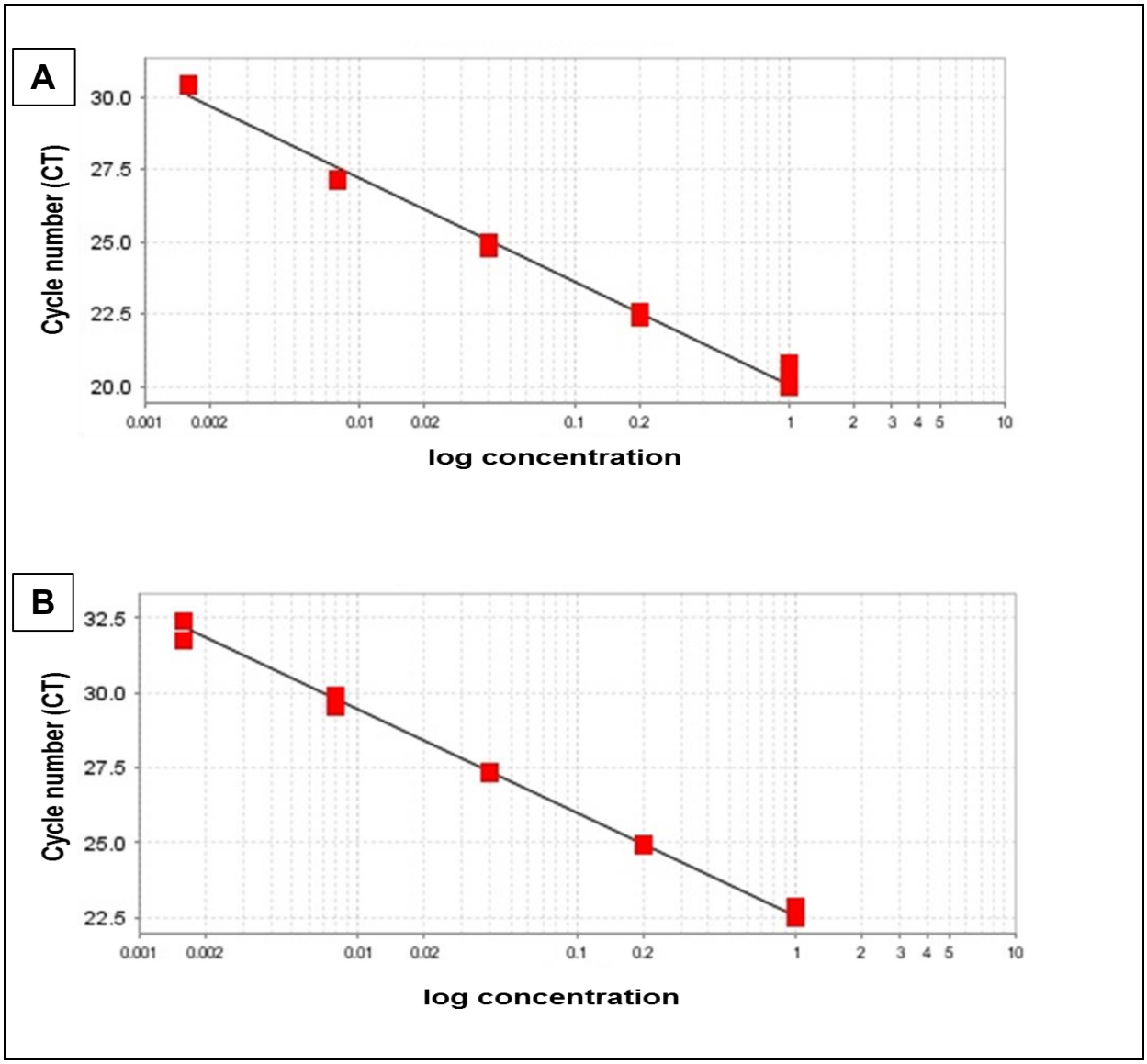
Determination of primer pair efficiency

To calculate the relative expression of the genes of interest by the method of (Pfaffl 2001), the primer efficiencies have to be determined. Primer efficiency is generally assumed to be two, since the product is doubled after every PCR cycle (Pfaffl 2001). To determine the primer efficiency, a serial dilution of a single cDNA sample was made, and each dilution was analysed in triplicate. A no template control was added, to determine if any contamination or primer self-amplification occurred. The samples were then analysed using the qPCR protocol described in section 2.7 of Chapter 2. A standard curve was generated using the StepOne™ software by plotting the cycle number (CT) (Y-axis) for each dilution against the log of the cDNA concentration (X-axis) (figure A2). The slope obtained from the standard curve is then used to

calculate the exponential amplification value (E), which is the primer efficiency for each primer pair, by using the following equation (Pfaffl 2001).

$$E = 10^{(-1/slope)} \qquad \text{(Equation 1)}$$

The primer efficiencies were 1.96, 1.94 and 2.12 for PR, PSA and GAPDH, respectively.



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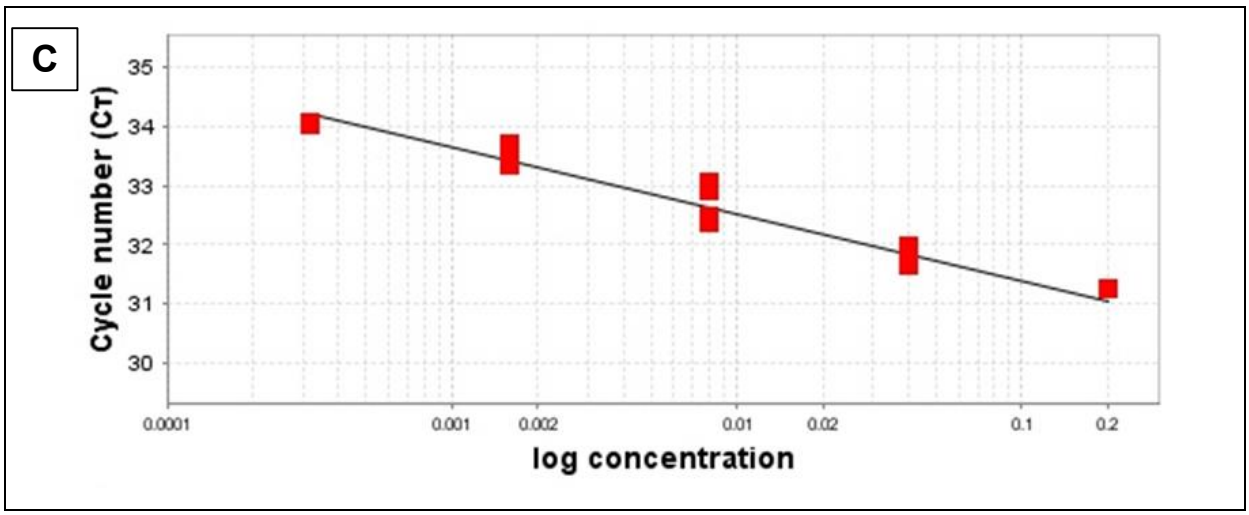
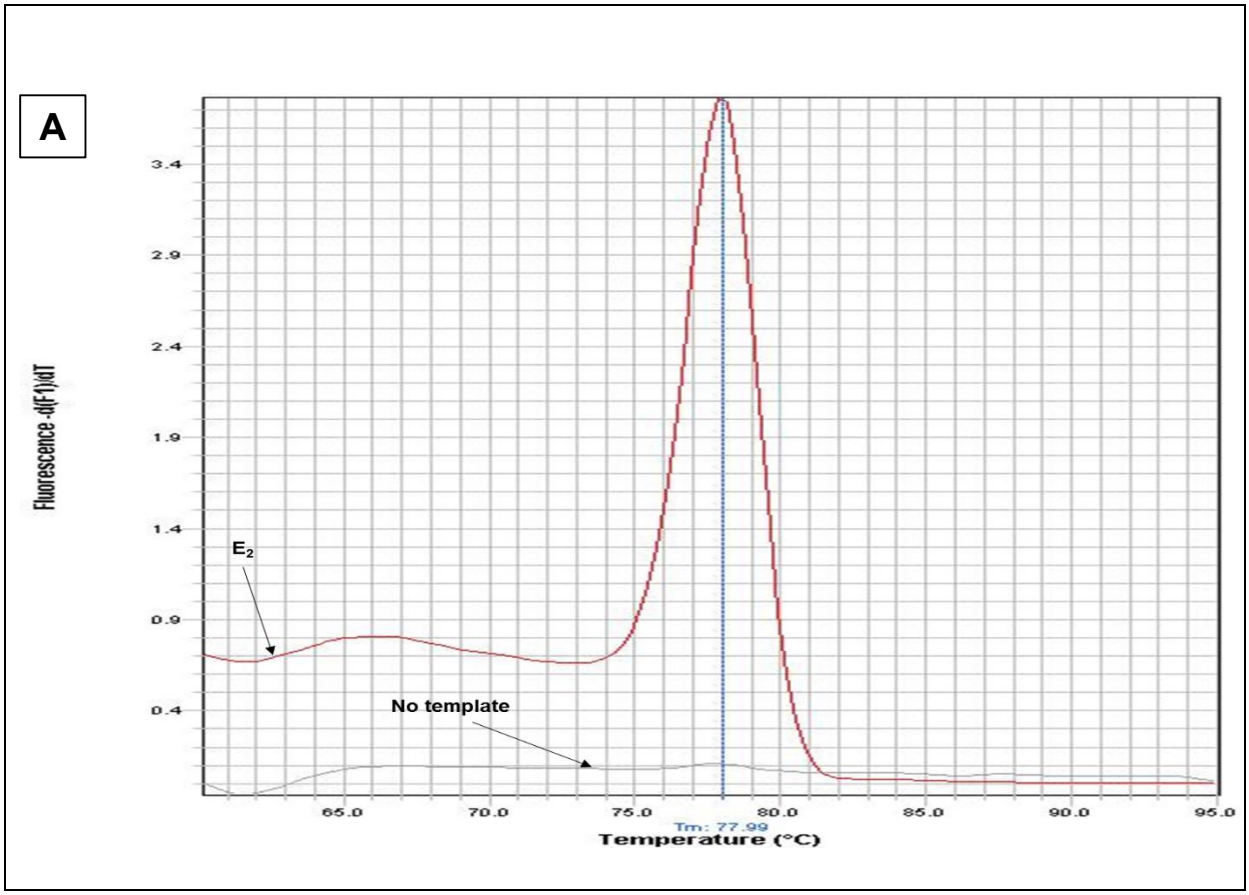


Figure A2. Representative standard curve showing cycle number (CT) versus the log DNA concentration for the (A) PR, (B) PSA and (C) GAPDH genes, respectively. The slope obtained from the each graph was used to calculate the primer efficiency.

Melting curve analysis

Melting curves are used as a way of optimization to confirm primer specificity, to determine whether there are any non-specific products and primer dimers. Primer dimers are indicated by the presence of small peaks that protrude from the product peak, while non-specific products can be indicated by the presence of peaks that deviate from the melting temperature (T_m) line (indicated by the vertical blue line in figure A3). During the PCR reaction SYBR green forms complexes with the double stranded DNA products. Therefore the fluorescence measured using SYBR green is the measurement of the sum of the relative levels of all products formed during the reaction. It is very important to ensure that the measured fluorescence is of a single product. This is done by increasing the temperature of the samples by 0.2^{o}C per second until the product's denaturation temperature of 95^{o}C is passed. The melting curve of a PCR product is dependent on the GC/AT content, length and sequence of the PCR product (Ririe *et al.* 1997). A melting curve is generally a plot of fluorescence as a function of temperature, and one product is indicated by one peak of high amplitude on the melting curve (Ririe *et al.* 1997). Examples

of melting curves for PR, PSA and GAPDH, respectively are shown below in figure A3, and a 2% agorose gel was used to further confirm that a single amplicon of the correct size was amplified (figure A4 (A-C)).



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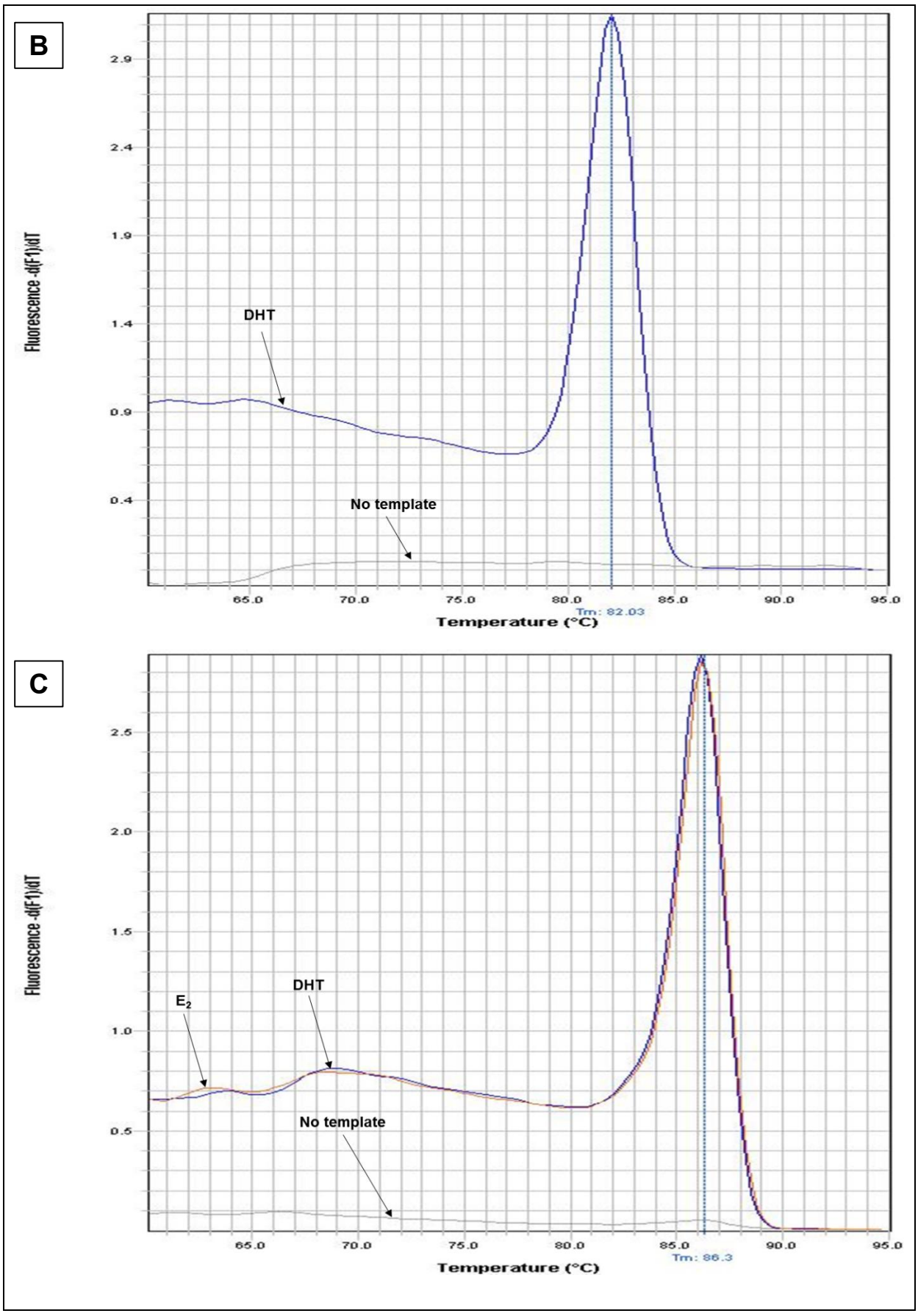

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Figure A3. Representative melting curves for the PR, PSA and GAPDH genes, respectively. MDA-MB-231 cells were transfected with (A and C) pSG5-ERβ and subsequently treated with 1 nM E_2, while MDA-MB-231 cells transfected with (B) $pSVAR_o$ were treated with 1 nM DHT. The red line represents samples treated with 1 nM E_2, the blue line indicates samples treated with 1 nM DHT and the grey line is the no template control.

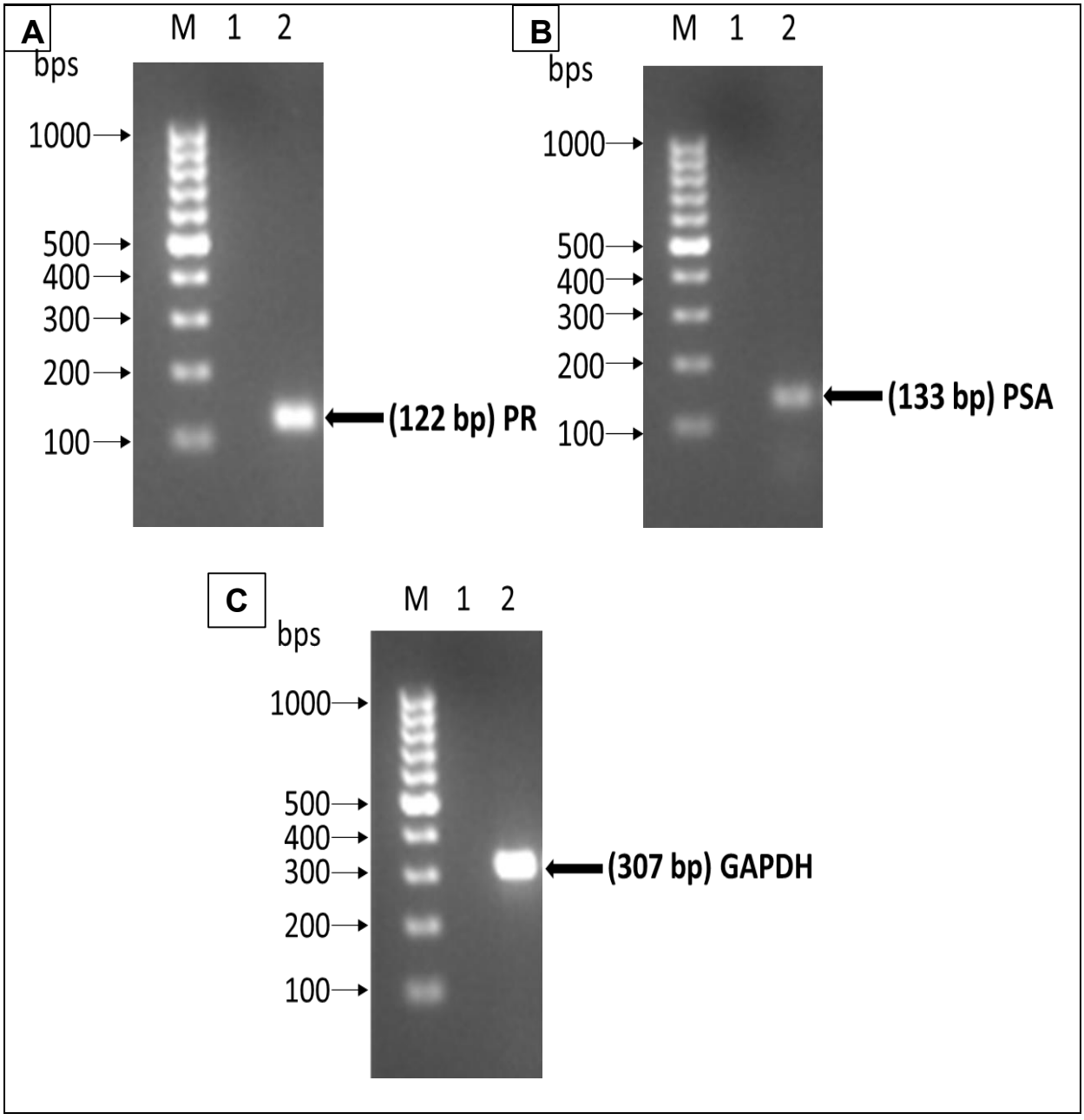

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Figure A4 Representative agarose gels showing qPCR products of (A) PR, (B) PSA and (C) GAPDH. Human MDA-MB-231 breast cancer cells were transfected with 2.5 ng **(A) and (C)** pSG5-hERβ or **(B)** $pSVAR_O$ using X-tremeGENE HP DNA transfection reagent according to the manufacturer's instructions for 24 hours. Cells were subsequently treated with 1 nM E_2 (PR and GAPDH) or DHT (PSA) for 12 hours. Total RNA was isolated, cDNA synthesized and mRNA expression levels of PR, PSA and GAPDH were determined by qPCR. The products were electrophoresed on a 2% agarose gel and visualized using ethidium bromide staining. Lane 1 represents the no template control, while lane 2 represents the single PCR amplicon of the correct size.

Determination of relative gene expression values

Having determined that a single amplicon of the correct size had been amplified, the relative expression (R) levels of the gene of interest were calculated using the mathematical model below (Pfaffl 2001), and expressed relative to the expression levels of the vehicle control (EtOH)

$$R = \frac{(E_{\text{target gene}})^{\Delta CP(\text{contol-sample})}}{(E_{\text{reference gene}})^{\Delta CP(\text{control-sample})}} \qquad \text{(Equation 2)}$$

The crossing point (CP) is the point in the PCR cycle where the target amplicon is first detected, and can also be referred to as the cycle number (C_t). C_t is used in ABI Prism® literature (Applied Biosystems, USA), while CP is used in LightCycler® literature (Roche Applied Science, USA) (Wong and Medrano 2005). The ΔCP is calculated for both the target and reference genes (GAPDH), by subtracting the CP value of the treated sample from the CP value of the vehicle control (EtOH) (Pfaffl 2001). A ratio of one indicates that there is no difference between the treated sample and that of the vehicle control. Results of experiments are graphically presented by plotting the cycle number versus the log concentration of the amplified cDNA.

Determining the optimal time for gene expression

MDA-MB-231 cells were transfected with 2.5 ng of either ERα, ERβ, AR or the pGL2 basic empty vector for 24 hours. The cells transfected with ERα, ERβ or pGL2 basic were treated with 0.1% EtOH or 1 nM E_2, while cells transfected with the AR were treated with 0.1% EtOH or 1 nM DHT for 2, 6, 12 or 24 hours. As shown in figure A5 A, E_2 did not upregulate the expression of the PR gene at any of the four time points in cells transfected with the empty vector. In contrast, cells transfected with ERα or ERβ showed significant E_2-induced upregulation of the PR gene (figure A5 B and C). Similarly, significant DHT-induced upregulation of the PSA gene was seen in cells transfected with the AR (figure A5 D). As good induction of both the PR (4.5-fold in the presence of ERα; 3.5-fold in the presence ERβ) and PSA genes (2.1-fold in the presence of the AR) were observed at 12 hours, this treatment time was chosen for all subsequent experiments.

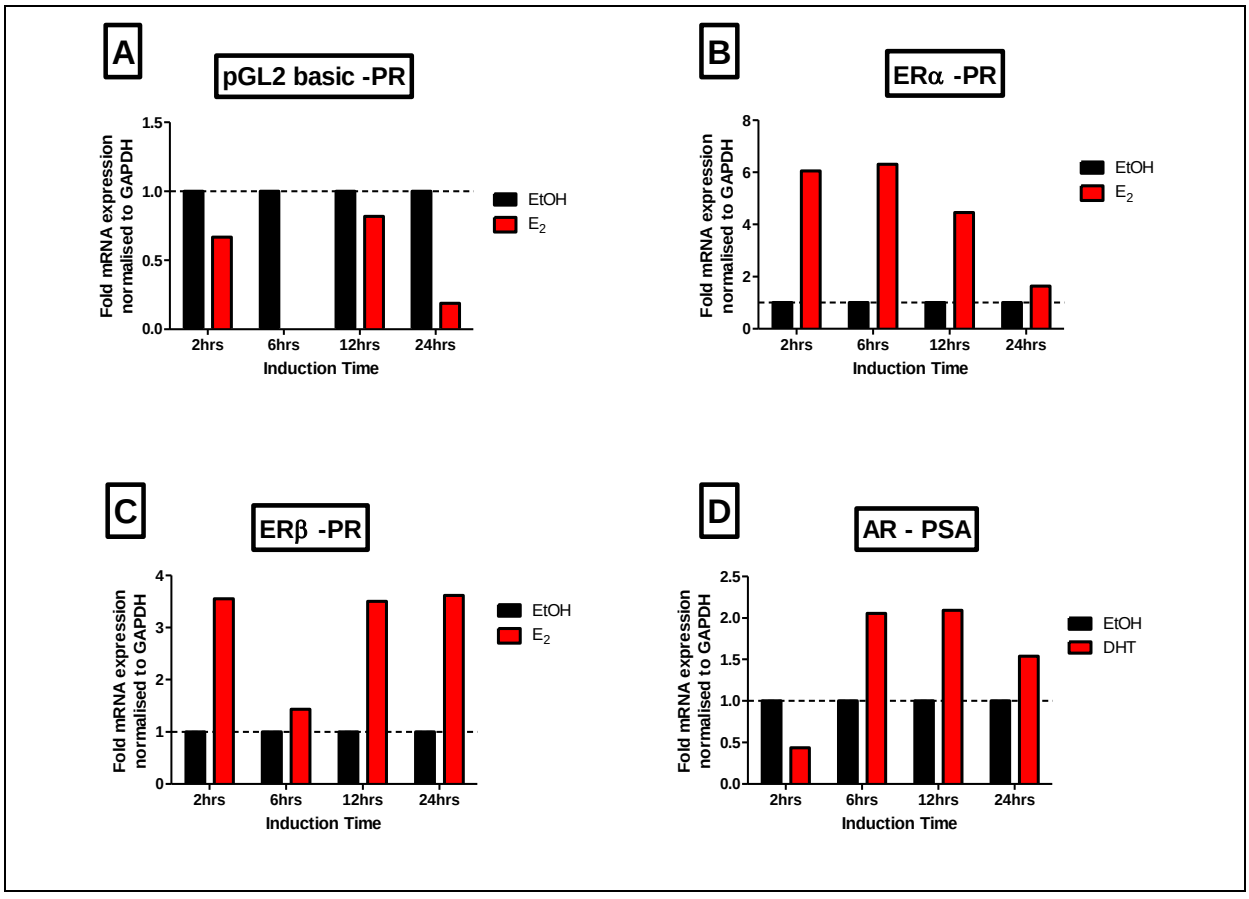

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Figure A5. Optimal gene expression for human PR and PSA. Human MDA-MB-231 breast cancer cells were transfected with 2.5 ng **(A)** pGl2 basic or **(B)** pSG5-hERα or **(C)** pSG5-hERβ or **(D)** pSVAR$_O$ using X-tremeGENE HP DNA transfection reagent according to the manufacturer's instructions for 24 hours. Cells were subsequently treated with 0.1% (v/v) EtOH (control) or 1 nM E_2 (PR) or DHT (PSA) for 2, 6, 12 and 24 hours. Total RNA was isolated, cDNA synthesized and mRNA expression levels of PR, PSA and GAPDH were determined by QPCR. GAPDH was used as the internal standard and the ratio of PR or PSA mRNA expression/GAPDH mRNA of all samples were calculated relative to the sample treated with 0.1% (v/v) EtOH. Results indicate the PR mRNA expression at 1 nM E_2 via **(A)** pGl2 basic or **(B)** ERα or **(C)** ERβ**. (D)** Results indicate the PSA mRNA expression at 1 nM DHT via AR.